IN VITRO MODELLING OF BACTERIAL POPULATION SHIFTS IN ORAL BIOFILMS.

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January 2008

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Thesis submitted for the degree of

Doctor of Philosophy

in

Clinical Dentistry (Microbiology)

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DECLARATION

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where otherwise stated.

Loading of sequencing reactions onto the sequencer was carried out by Dr. Adam Roberts and Miss Tracey Moss (UCL Eastman Dental Institute, London, UK).

The formulation of hydroxyapatite discs with silver incorporated was carried out by Dr. George Georgiou (UCL Eastman Dental Institute, London, UK).

ABSTRACT

In vitro models of dental plaque are a valuable tool for understanding the development of plaque-related diseases and assessing potential treatments for these diseases. The main focus of this study was the development of an *in vitro* model to characterise the changes in bacterial populations from dental plaque associated with health to one associated with gingivitis.

By emulating environmental conditions in the oral cavity associated with gingivitis it was possible to see changes in the oral microbiota associated with gingivitis. Using traditional culture techniques the ascendancy of *Actinomyces* spp. at the expense of *Streptococcus* spp. was observed with the onset of gingivitis conditions, along with increased proportions of Gram-negative species.

To assess the range of cultivable species present isolates, which had previously been cultured in the model, were identified by sequencing of the 16S rRNA gene. After the onset of gingivitis conditions a greater richness of species was identified. Examination of these communities with confocal microscopy and viability staining also revealed structural changes associated with environmental conditions emulating gingivitis.

To assess the presence of species which were not frequently identified by culture, but previously shown to be associated with gingivitis, quantitative PCR (qPCR) was used to enumerate *Prevotella* spp., *Fusobacterium* spp. and *Porphyromonas gingivalis*. *Prevotella* and *Fusobacterium* spp. were found to be significant members of the microbial communities developed in the CDFF, with *Prevotella* spp. increasing significantly under conditions emulating gingivitis. Furthermore, the total bacterial counts enumerated by culture were underestimated by approximately 80% compared to the total counts obtained by qPCR.

This model was ultimately used to assess the effectiveness of tetracycline, chlorhexidine and silver ion-releasing dental materials against the accumulation of plaque. All of these agents influenced the microbial composition, rather than total microbial numbers, with reduced levels of *Actinomyces*, *Prevotella* and *Fusobacterium* spp.

This study has shown that i*n vitro* models of microbial communities associated with health and disease are valuable tools for observing key factors in disease progression. When disease results from changes in the resident microflora the use of such models allows the influence of individual environmental factors to be assessed and also allows the effect of potential treatments on these communities to be examined.

ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge the EPSRC and Proctor and Gamble for funding this project.

I would like to especially thank my supervisors Dr. Jonathan Pratten and Dr. Dave Spratt for their encouragement and guidance over the course of my PhD.

I would also like to thank all the staff of the Division of Microbial Diseases, for their help, friendship and coffee breaks which helped me to survive my PhD. I would like to give a special thanks to Miss Emily Earsman for her help with qPCR and from the Division of Biomaterials and Tissue Engineering Dr. George Georgiou and Dr. Showan Nazhat for their advice and help in the production of the antimicrobial incorporated hydroxyapatite discs used for the last part of this project.

Last but not least I would like to thank my parents for their encouragement, support and patience and my sisters Aneesa and Sabiha for their constant support, understanding, proofreading services and keeping me motivated over the past four years.

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ABBREVIATIONS

ANUG	Acute Necrotising Ulcerative Gingivitis
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
BPA	Black Pigmenting Anaerobe
°C	Degree Celsius
CBA	Columbia Blood Agar
CBA-GN	Columbia Blood Agar with Gram-Negative supplement
CDFF	Constant Depth Film Fermenter
CFAT	Cadmium Fluoride Acriflavin Tellurite agar
CFU	Colony-forming units
CHX	Chlorhexidine
CLPP	Community Level Physiology Profiling
CLSM	Confocal Laser Scanning Microscopy
DGGE	Denaturing gradient gel electrophoresis
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EM	Electron Microscopy
EPS	Extracellular polysaccharide
FAA	Fastidious Anaerobe Agar
FISH	Fluorescent in-situ hybridisation
g	Gravitational Force
H	Hour
HA	Hydroxyapatite
kb	Kilobase
µl	Microlitre
µm	Micrometer
M	Molar
min	Minute
mL	Millilitre

mm	Millimetre
MS	Mitis-Salivarius
MSD	Multiple Sorbarod Device
μM	Micro-molar
nm	Nanometer
nM	Nanomolar
qPCR	Quantitative Polymerase Chain Reaction
pbp	Penicillin binding proteins
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
psi	Pounds per square inch
RA	Rogosa agar
rRNA	Ribosomal Ribonucleic acid
rpm	Revolutions Per Minute
S (16S, 23S)	Sedimentation coefficient
s	Second
SDS	Sodium Dodecyl Sulphate
spp.	Species (plural)
SSU	Small subunit
SYBR	SYBR green nucleic acid dye
TCH	Tetracycline Hydrochloride
Tm	Melting temperature
VA	Veillonella agar

INTRODUCTION

1.1 The Bacteria of the Oral Cavity

Of all life on earth microbial organisms display the greatest range of diversity. If we examine the range of organisms present in the three biological domains (Archaea, Bacteria and Eucarya, Fig. 1.1.1) the greatest diversity of species is observed within the bacterial group (Pace, 1997). The human body is colonised by bacteria within 24 hours of birth (Tannock, 1995) due to the transfer of bacteria to infants during birth and the rapid exposure to a vast array of microorganisms in the environment. The human body provides an abundance of surfaces for microbial colonisation including the skin, various surfaces in the oral cavity and the gastrointestinal tract. This microbial colonisation is both beneficial to the human host and the colonising microorganisms as their presence in a stable microbial community provides resistance to colonisation by more pathogenic microorganisms, whilst the host provides a stable environment in which these organisms can proliferate. Disruption of this stable host environment can cause bacteria, which form part of the normal microbiota associated with health, to be involved in the development of disease. The development of periodontal diseases is a classic example of this.

Figure 1.1.1: Universal phylogenetic tree based on SSU rRNA units. (From Pace, 1997).

1.1.1 Dental plaque development

The oral microbial community is dynamic and diverse. From initial plaque development to the development of periodontal diseases the microbial community changes as a result of bacterial succession based on changes in the oral environment.

There are several key stages in dental plaque development. The first process is the adsorption of host and bacterial molecules to the tooth surface, followed by attachment of bacteria to this surface. Further bacteria can then attach by co-adhesion to these early colonizers (Marsh, 2004). The microbial composition of these communities is then influenced by changes in environmental parameters which will often encourage the growth of particular species.

1.1.1.1 Early colonisation

Early colonisers of dental plaque are organisms which are able to survive in the high oxygen environment and are able to adhere to the surfaces available for colonisation in the oral cavity. Adsorption of salivary proteins, peptides and other organic molecules from the saliva form a proteinaceous film on the tooth surface known as the acquired enamel pellicle. Initial colonisers of dental plaque are able to bind to these salivary components. Saliva has been shown to enhance the adherence of *Actinomyces naeslundii* and *Streptococcus gordonii* cells to a surface (Palmer *et al*, 2001). Based on microscopic studies of plaque development the initial colonisers are predominantly coccoid organisms in conjunction with a few rod-shaped species. Two major genera involved in the initial attachment to the pellicle of the teeth are *Actinomyces* and *Streptococcus* species. The Type I fimbrae of *Actinomyces viscosus* species are able to bind to proline-rich proteins present in the acquired pellicle (Gibbons & Hay, 1988). Species of *A. naeslundii* also produce neuraminidases which interact with salivary

glycoproteins (Qureshi & Gibbons, 1981) and thus serve a role in initial adherence. *Streptococcus sanguinis* (formerly *S. sanguis*, Ciardi *et al*, 1987), *S. parasanguinis* and *S. gordonii* have all been shown to bind to saliva-coated hydroxyapatite through adhesins.

Streptococcus spp. account for between 47 and 85 % of the bacteria detectable by culture 4 hours after professional tooth cleaning (Nyvad & Kilian, 1987). *Actinomyces* spp. were found to account for 50% of detectable species using checkerboard DNA hybridisation on the first day of plaque development (Ramberg *et al*, 2003), being the dominant genus at 6 hours (Li *et al*, 2004). *Streptococcus* spp. proportions increase significantly over this time whilst the relative numbers of *Actinomyces* spp. remain the same. Again, it was observed that *Streptococcus* spp. were the dominant genera after the initial attachment stage (Ramberg *et al*, 2003). Other frequently observed genera in early plaque communities include *Gemella*, *Granulicatella*, *Neisseria*, *Prevotella*, *Rothia* and *Veillonella* spp. (Diaz *et al*, 2006).

Using molecular techniques it is possible to detect a greater range of oral species in developing plaque than by using traditional culture techniques. Using culture techniques alone the most commonly isolated species in developing plaque was found to be *S. sanguinis*, with *A. viscosus*, *Streptococcus mitis*, *Staphylococcus aureus*, *Peptostreptococcus* spp. and *Veillonella* spp. also frequently being detected (Socransky *et al*, 1977). In contrast, using checkerboard DNA-DNA hybridization, Socransky *et al*, (1994) found *Streptococcus oralis* and *S. mitis* to be the predominant early colonisers of dental plaque. *A. naeslundii*, *S. gordonii* and *Eikenella corrodens* were also significant members of these populations. Interestingly periodontal pathogens such as *Trepomena*

denticola, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were also detected at very low levels in these populations. Identification of different 16S rRNA sequence phylotypes present in dental plaque samples (Palmer *et al*, 2001; 2003) has shown that apart from the *Streptococcus* and *Actinomyces* spp. and there were several other genera present in significant proportions in the developing dental plaque including *Gemella*, *Granulicatella*, *Neisseria*, *Prevotella*, *Rothia* and *Veillonella* species. Again organisms considered to be late colonisers, which may influence disease progression, such as *Porphyromonas* spp. could be detected at low levels in these communities.

1.1.1.2 Importance of bacterial interactions in the formation of oral biofilms

Bacteria growing as part of a biofilm are involved in a wide range of physical, metabolic and molecular interactions with neighbouring bacteria (Marsh, 2005). The ability of early colonisers to adhere to each other, forming specific co-aggregation groups is important in plaque development. Strains of *A. viscosus* and *A. naeslundii* are found to co-aggregate strongly with oral strains of *S. mitis* and *S. sanguinis* (Cisar *et al*, 1979) using a variety of cell surface interactions. Furthermore, early colonizing *Streptococcus* spp. can act as receptors for the attachment of further species. For example, the adhesins produced by *S. gordonii* and *S. sanguinis* mediate co-aggregation with *A. naeslundii* (Kolenbrander, 1993). *S. gordonii* also demonstrate binding properties to salivary proteins as a mechanism for attachment to the tooth surface. Using an *in vitro* model (Palmer *et al*, 2001) *S. gordonii* were able to grow as monoculture biofilms but *S. oralis* and *A. naeslundii* were not. In co-culture with *S. gordonii* they also displayed little growth. When grown together however they displayed significant growth, showing more growth than the single species biofilms of *S. gordonii* suggesting

that growth in combination with other species is advantageous in complex microbial communities as opposed to growth as a single species. Another example are *Veillonella* spp. which are unable to form biofilms on the tooth surface alone (Liljemark & Gibbons, 1971) but in combination with oral streptococci grow in significant numbers (McBride & Van der Hoeven, 1981). This is due to the organic acids produced by *Streptococcus* spp. as they breakdown sugars being used by *Veillonella* spp. (Mikx *et al*, 1972).

1.1.1.3 Bacterial succession in dental plaque development

Initial colonization of the tooth surface is by aerobic and aerotolerant bacteria. As plaque matures anaerobic and less aerotolerant species become more prevalent (Marsh & Martin, 1999). The attachment of late colonizers is dependent on their attachment to early colonizers. Some of these interactions and the sequence of bacterial attachment and succession are summarised in Fig. 1.1.2.

Figure 1.1.2: Bacterial attachment and succession.

(From Kolenbrander *et al*, 2002)

Fusobacterium nucleatum displays coaggregative properties with both early and late colonizers and is thus thought to have a bridging role in dental plaque between species (Bolstad *et al*, 1996). It is the most common Gram-negative organism isolated from healthy plaque and its numbers are known to increase as periodontal diseases develop (Slots *et al*, 1978; Moore & Moore, 1994). Typical morphology associated with the presence of fusobacteria is the formation of corncob structures (Fig. 1.1.3) when these organisms aggregate with other species (Lancy *et al*, 1983; Kolenbrander *et al*, 1989).

The presence of these formations is indicative of mature plaque structure (Listgarten, 1994).

Figure 1.1.3: Corncob formations in dental plaque.

(From Takeuchi & Yamamoto, 2001).

The bridging role of *F. nucleatum* is essential for the attachment of organisms associated with mature plaque (Kolenbrander *et al*, 1993; Kolenbrander *et al*, 2006). *Selenomonas flueggei* co-aggregates with *F. nucleatum* but not with early colonizers of dental plaque (Kolenbrander, 1989). *Eubacterium* spp. have also been shown to co-aggregate with strains of *F. nucleatum* but not with *A. actinomycetemcomitans*, *P. gingivalis* or *Prevotella intermedia* (George & Falkler, 1992). Bradshaw *et al*, (1998) showed that in a ten species model of oral biofilms *F. nucleatum* played a key role in the survival of strictly anaerobic species such as *P. gingivalis* and *Prevotella nigrescens* in mixed, aerated planktonic cultures. When *F. nucleatum* was omitted from these cultures the presence of these species was significantly reduced. *F. nucleatum* was

found to co-aggregate strongly with all other species used in the model suggesting that *F. nucleatum* plays a key role as a bridge between aerobic and strictly anaerobic species. Other Gram-negative species associated with periodontal disease such as *T. denticola* and *P. gingivalis* are thought to be associated with *F. nucleatum*, possibly being an essential requirement for their colonization. *F. nucleatum* possesses the ability to bind to host-derived proteins but does not possess the proteolytic activity to use them as a nutrient source. When *F. nucleatum* is bound to proteolytic species, such as *P. gingivalis*, their proteolytic activity can be used to break down these bound proteins, forming a mutually beneficial relationship to both bacteria (Rogers *et al*, 1998). Co-aggregation bridges are also important in bringing together species which would not co-aggregate with each other. For example *Prevotella loescheii* acts as a bridge between *S. oralis* and *Actinomyces israelii* (Kolenbrander *et al*, 2006). *T. denticola* is known to co-aggregate with *P. gingivalis* and in sites where no *P. gingivalis* was detected *T. denticola* was also not detected (Grenier, 1992a). This indicates that *P. gingivalis* may be necessary for the attachment of this organism or that both require the same environmental conditions (e.g. Eh) to become established. Co-aggregation of obligately anaerobic species to oxygen consuming species can ensure their survival in an aerobic environment (Bradshaw *et al*, 1998).

1.1.2 The range of microbial species in dental plaque

The range of bacterial genera that have been identified in the oral cavity are summarized in Table 1.1.1. The role of all species, particularly those that have only recently been identified or are new candidate divisions, is still to be fully elucidated.

Oral phyla	Genus	Isolated in the Oral cavity	References
Firmicutes	***Abiotrophia***	Early dental plaque	Mikkelsen *et al*, 2000
	Catonella	Gingival crevice	Paster *et al*, 2001
	Centipeda	Periodontal lesions	Lai *et al*, 1983
		Subgingival plaque	Sawada *et al*, 2000; Siqueira Jr. & Rocas, 2004
	Dialister	Periodontal disease	Contreras *et al*, 2000
	Enterococcus	Advanced periodontitis	Rams *et al*, 1992
		Periapical lesions	Sundqvist *et al*, 1998
	Eubacterium	Periodontitis	Moore *et al*, 1993; Paster *et al*, 2001; Kumar *et al*, 2003
	Filifactor	Periodontal diseases	Cato *et al*, 1985; Kumar *et al*, 2005
	Gemella	Early dental plaque	Mikkelsen *et al*, 2000
	Granulicatella	Oral cavity	Aas *et al*, 2005; Paster *et al*, 2006
	Lactobacillus	Normal microbiota	Sutter, 1984
		Root surface caries	Shen *et al*, 2004
	Peptostreptococcus	Periodontal diseases	Slots *et al*, 1978; Kremer *et al*, 2000; Riggio & Lennon, 2003
	Pseudoramibacter	Oral cavity	Downes *et al*, 2001
	Selenomonas	Periodontitis	Dzink *et al*; 1988; Tanner *et al*,1998
	Solobacterium	Oral cavity	Paster *et al*, 2001; Rolph *et al*, 2001

	Streptococcus	Early coloniser	Socransky *et al*, 1977; Beckers & Van der Hoeven, 1982; Nyvad & Killian, 1987
		Dominant genus at various locations in the oral cavity	Krasse, 1954; Mager *et al*, 2003; Aas *et al*, 2005
		Caries	Loesche *et al*, 1975; Loesche *et al*, 1986; Nyvad & Killian, 1990
	Veillonella	Caries	Noorda *et al*, 1988; Becker *et al*, 2002
Fusobacteria	***Fusobacterium***	Developing plaque	Loe *et al*, 1965b; Socransky *et al*, 1977; Kolenbrander *et al*, 2006
		Co-aggregation	Lancy *et al*, 1983; Kolenbrander *et al*, 1989; Bradshaw *et al*, 1998; Rickard *et al*, 2003
	Leptotrichia	Subgingival plaque	Kasai *et al*, 1965; Socransky, 1970
Actinobacteria	***Bifidobacterium***	Normal microbiota	Evaldson *et al*, 1982; Sutter *et al*, 1984; Crociani *et al*, 1996
	Actinomyces	Early coloniser	Ellen, 1978; Sarkonen *et al*, 2000
		Gingivitis	Syed & Loesche, 1978; Moore *et al*, 1982; 1984; 1987
	Corynebacterium	Subgingival dental plaque	Kumar *et al*, 2003
	Propionibacterium	Subgingival plaque	Williams *et al*, 1979
	Rothia	Normal microbiota	Ishikawa, 1980; Nyvad & Killian, 1987; Aas *et al*, 2005

	Mogibacterium	Oral cavity	Nakazawa *et al*, 2000
	Slackia	Periodontitis	Wade *et al*, 1999
	Olsenella	Human gingival crevice	Olsen *et al*, 1991
Spirochaetes	***Treponema***	Subgingival microbiota	Moore *et al*, 1987; Dahle *et al*, 1993
Proteobacteria	***Actinobacillus***	Aggressive periodontitis	Slots *et al*, 1999Ebersole *et al*, 1982; Zambon *et al*, 1983; Novak & Novak, 1996; Slots *et al*, 1999
	Campylobacter	Subgingival plaque of health and periodontal disease	Slots *et al*, 1978; Tanner *et al*, 1979; Socransky *et al*, 1998; Paster *et al*, 2001
	Cardiobacterium	Oral cavity	Sixou *et al*, 1996
	Desulphobacter/ Desulphovibrio	Subgingival plaque	Van der Hoeven *et al*, 1995
	Eikenella	Subgingival plaque	Chen *et al*, 1989; Chen & Wilson, 1992; Aas *et al*, 2005
	Haemophilus	Subgingival plaque	Slots *et al*, 1978; Tanner *et al*, 1994
	Helicobacter	Plaque and saliva	Nguyen *et al*, 1995; Gebara *et al*, 2004
	Kingella	Oral cavity	Chen, 1996
	Moraxella	Opportunistic pathogen found in the oral cavity	Senpuku *et al*, 2003
	Neisseria	Commensal organism	Liljemark & Gibbons, 1971; Long & Swenson, 1976

Bacteroidetes	***Bergeyella***	Oral cavity	Han *et al*, 2006 (uncultivated strain)
	Capnocytophaga	Mature plaque	Moore *et al*, 1987; Ciantar *et al*, 2001; Aas *et al*, 2005
	Porphyromonas	Periodontal pathogen	Frisken *et al*, 1987; Socransky *et al*, 1988
	Prevotella	Normal microbiota	Zambon *et al*, 1981; Shah & Collins, 1990
		Periodontal diseases	Syed & Loesche, 1978; White & Mayrand, 1981; Lie *et al*, 2001
	Tannerella	Periodontal pockets	Tanner *et al*, 1979; Genco, 1996
Deferribacteres	***Deferribacteres***	Subgingival plaque chronic periodontitis	Kumar *et al*, 2003
		Endodontic infections	Siqueira *et al*, 2005
			Paster *et al*, 2001; Kumar *et al*, 2003; Aas *et al*, 2005
Candidate divisions	***TM7***	Subgingival plaque	
		Tongue dorsa	Kazor *et al*, 2003
	OP11	Subgingival plaque	Paster *et al*, 2001; Kumar *et al*, 2003

Table 1.1.1: The microbial inhabitants of the oral cavity.

Key oral bacterial species have been grouped together into clusters associated with specific stages of plaque development and disease progression (Table 1.1.2). Organisms belonging to the same cluster tend to be isolated together in relation to the development of periodontal disease (Socransky *et al*, 1998) and it has been inferred that bacteria from the same cluster have similar nutritional and atmospheric requirements as they are occupying the same environmental niche.

Table 1.1.2: Bacterial complexes formed in subgingival plaque.

(From Socransky *et al*, 1998)

Bacteria in the yellow complex are associated with periodontal health and early plaque development. Species from the green cluster are associated with maturing plaque, while species from the orange and red clusters tend to be associated with subgingival plaque

and disease. Specific clusters also interact with each other. For example, species belonging to the red complex are rarely isolated without the presence of species from the orange complex (Socransky *et al*, 1998).

1.1.2.1 Distinction between supra and subgingival plaque

Figure 1.1.4: Distinction between supra and subgingival plaque. o Represent Gram-positive cocci, ▭ represent Gram- positive rods, ▭ represent Gram-negative rods, ~~~ represent motile bacteria.

(Adapted from www.dent.umich.edu/.../loeschelabs/pocket.gif).

Supra and subgingival plaque are distinct in their location, composition and structure. Supragingival plaque forms above the gingival margin (Fig. 1.1.4). During initial colonisation bacterial colonies of mainly coccoid bacteria spread laterally across the tooth surface. Further bacterial proliferation, upwards from the tooth surface occurs by

the formation of tightly packed columns (Listgarten, 1994). Filamentous bacteria begin to attach at this stage which is visualised by the formation of corncob structures. The supragingival plaque of healthy gingiva will tend to be dominated by Gram-positive species, Gram-negative rods and filaments while spirochaetes become more significant members of these communities as plaque accumulates and matures (Fig. 1.1.5).

Figure 1.1.5: Morphologies observed in supragingival plaque. A) Early plaque formation, dominated by cocci; B) Interactions between filamentous and coccoid bacteria. SEM images. Bar = 4 μm (From Takeuchi & Yamamoto, 2001).

Plaque which forms below the gingival margin in the gingival sulcus is defined as subgingival plaque (Fig. 1.1.4). This sulcus provides an anaerobic environment and thus the bacteria that colonise this area have different atmospheric requirements than those of the supragingival plaque. In contrast to the supragingival plaque, bacteria form a thin adherent layer on the tooth surface. The bacteria that tend to dominate in this

environment include spirochaetes and motile rods (Fig. 1.1.6). Gingival tissues are also in contact with bacterial cells and may also serve as surfaces for attachment.

Figure 1.1.6: Morphologies typical of subgingival plaque.

A), Test-tube brush formations surrounded by motile, Gram-negative bacteria, Magnification x 1700; B Spirochaetes with their distinctive ultrastructure. Bar = 0.5 μm (From Listgarten, 1994).

1.1.2.2 The increase in species richness as plaque develops

Most studies aiming to define the microbial composition of dental plaque have looked at the richness or abundance of species present. Many of the early studies into the oral microbiota have relied on culture techniques and even with exhaustive identification procedures of isolates on selective and non-selective media it is simply not possible to grow every single oral microorganism *in vitro*. Also since the initial studies on the richness of oral organisms present in dental plaque there have been many new species discovered and some reclassification of existing species.

In order to understand the role of particular bacterial species in periodontal diseases it is first necessary to define the normal flora of the oral cavity. Defining this flora is complicated by the sheer number of species which have been isolated from the oral cavity and the site-specific communities that develop at different locations on different

surfaces. Identifying the richness of species present in the healthy oral cavity is an ongoing task due to the large proportion of species which have not yet been cultivated. Aas *et al*, (2005) used culture-independent techniques to examine the diversity of species present in the healthy human oral cavity at different sites. Based on the analysis of over 2,500 16S rRNA clones they identified 141 different bacterial taxa belonging to six different bacterial phyla. These included species belonging to the *Firmicutes* (e.g. *Streptococcus*, *Gemella*, *Eubacterium*, *Selenomonas*, *Veillonella*), the *Actinobacteria* (e.g. *Actinomyces*, *Atopobium*, *Rothia*), the *Proteobacteria* (e.g. *Neisseria*, *Eikenella*, *Campylobacter*), the *Bacteroidetes* (e.g. *Porphyromonas*, *Prevotella*, *Capnocytophaga*), the *Fusobacteria* (e.g. *Fusobacterium*, *Leptotrichia*) and TM7 phylum (unculturables). Over 60% of these phylotypes represented organisms which had not previously been cultivated. On the tooth surface the most commonly detected genus was *Streptococcus* spp. with *Rothia dentocariosa*, *Gemella haemolysans*, *Granulicatella adiacens*, *Actinomyces* sp. clone BL008, and *Abiotrophia defectiva* often being detected. Interestingly using this technique the periodontal pathogens *P. gingivalis*, *T. forsythia* and *T. denticola* were not detected from any site which indicates that species present at low levels are not picked up using this method. Using alternative assessment techniques and a much larger sampling group these organisms have been detected as part of the normal flora of supragingival plaque of healthy patients (Ximenez- Fyvie *et al*, 2000c) but increased proportions were observed in subgingival samples from periodontitis patients.

Anaerobes form a significant part of the flora of the oral cavity, including species from the following genera, *Actinomyces*, *Prevotella*, *Bifidobacterium*, *Eubacterium*, *Fusobacterium*, *Lactobacillus*, *Leptotrichia*, *Peptostreptococcus*, *Propionibacterium*,

Selenomonas, *Treponema*, and *Veillonella* (Sutter, 1984). These bacteria can form significant portions of the microbiota of healthy sites.

A common finding from studies of the oral microbiota of different individuals is that there is great diversity in the composition of the oral microbiota between individuals. Periodontal disease development may be a subject-specific process, severity of disease being dependent on the initial plaque composition. Retention of higher numbers of periodontal pathogens in non-diseased plaque may be influenced by specific environmental factors in individuals such as dietary factors or the use of antimicrobials which may influence the diversity of plaque composition between individuals and the difference in response to experimental gingivitis. Specific sites within the mouth may harbour these pathogens such as interdental regions of plaque. These areas have been found to be a potential habitat for *A. actinomycetemcomitans*, *Pr. intermedia*, *Pr. nigrescens*, *Campylobacter rectus*, *P. gingivalis* and *Bacteroides forsythus* (*T. forsythensus*) (Gmur & Guggenheim, 1994).

1.2 The Oral Environment

The oral cavity is an environment which supports the growth of complex microbial populations. Several environmental niches can develop within the oral cavity and thus organisms with very different growth requirements can exist within the same ecosystem. The high richness of species in the oral cavity is due to a variety of environmental factors which can change depending on the site sampled, the individual or health status.

1.2.1 Environmental factors influencing the oral ecosystem

1.2.1.1 Temperature

The temperature of the oral cavity remains relatively stable between 35 and 36°C (Moore *et al*, 1999), fluctuating during the consumption of food and beverages (Marcotte & Lavoie, 1998) and at different sites in the oral cavity (Moore *et al*, 1999). This temperature range is optimal for the growth of many microorganisms.

1.2.1.2 pH

Saliva maintains the pH of the oral cavity near neutral (Mandel, 1987), the pH of saliva remaining in the region of pH 6.75 and 7.25 (Marsh & Martin, 1999). The flowing action helps to remove potential substrates such as carbohydrates from the oral environment (Humphrey & Williamson, 2001) and a buffering effect is provided by bicarbonate (HCO_3^-), peptides, proteins and phosphates present in the saliva (Edgar & Higham, 1995; Bardow *et al*, 2000). The effectiveness of these systems is highly dependent on the flow rate of saliva (Tenovuo, 1997), whether saliva is stimulated or unstimulated. The HCO_3^- concentration is higher in stimulated saliva whilst phosphate ions are more concentrated in unstimulated saliva (Ferguson & Fort, 1973). The buffering capacity of salivary proteins is thought to be related to the pH being below or

above their isoelectric point, which for most salivary proteins is in the physiological pH range and thus this buffering system is thought to be most important when the pH is either acidic or alkaline (Bardow *et al*, 2000).

The main cause of fluctuations in pH is the intake of sugary foods which causes the pH to become more acidic (Grossman & Brickman, 1937) and favours the growth of caries-causing microorganisms such as *Lactobacillus* spp. (Bowden, 1990) and *Streptococcus mutans* (Loesche, 1986). The fluctuations in pH associated with sugar intake are best described by a Stephan curve (Fig. 1.2.1).

Figure 1.2.1: Stephan curve

(From www.ncl.ac.uk)

Immediately after sugar intake there is a rapid decrease in pH from near neutral to an acidic pH (Stephan, 1944). The pH then gradually returns to the resting pH of near neutral, taking anywhere between 15 and 40 minutes depending on the individual (Dong *et al*, 1999). The level of pH reduction is dependent on the carbohydrate source (i.e. sources which are readily fermentable and those which are more complex and require breakdown in order to be metabolised (Frostell *et al*, 1967) and the microbial composition of plaque (Gibbons & Armstrong, 1964). The presence of high proportions

of aciduric and acidogenic bacteria combined with the intake of carbohydrates which can be easily metabolised can result in a pH as low as 4.5 (Brailsford *et al*, 2001). The flowing action of saliva is important in the return to resting pH. Saliva dilutes and washes away metabolites and salivary bicarbonates neutralise the acidic end products of carbohydrate metabolism. The gingival crevice pH is more alkaline (Bickel *et al*, 1985) as gingival crevicular fluid does not have the same buffering ability as saliva and may favour the growth of periodontopathogens. The mean pH of the gingival crevice is between pH 7.2 and 7.4 and the optimum growth rate for *P. gingivalis* is pH 7.5 (Marsh & Martin, 1999) thus making the pH of the GCF more hospitable for these organisms than saliva.

1.2.1.3 Oxidation-reduction potential

The availability of oxygen varies at different sites in the oral cavity allowing a wide range of species with different requirements to grow. As plaque develops, oxygen levels decrease due to the consumption of oxygen by facultative organisms and the reduction in diffusion of oxygen as plaque thickness increases. The oxidation reduction potential of the supragingival plaque drops significantly in the first few days of plaque development (Kenney & Ash, 1969). This is indicated by the increased presence of strict anaerobes as part of the oral microbiota over time. As plaque develops the microbial composition becomes stratified, anaerobic organisms are confined to deeper layers and aerobic organisms are present nearer the surface. This allows the growth of strict aerobes such as *Neisseria* spp. and that of obligate anaerobes such as *P. gingivalis* in the same biofilm (Kinniment *et al*, 1996). Oral streptococci associated with supragingival plaque have a high ability to metabolise oxygen molecules and defend against the toxic products of oxygen metabolism through the production of NADH

peroxidase and superoxide dismutase (SOD) (Marquis, 1995). High levels of SOD activity have been detected in the gingiva of patients with chronic periodontitis compared to controls (Akalin *et al*, 2005).

1.2.1.4 Nutrient source

The oral environment is a low nutrient environment. Growth as a biofilm allows what nutrients are available to be concentrated and helps retain solutes that might otherwise be lost from cells (Marquis, 1995). Organisms which are associated with a particular habitat within the mouth are usually associated with the nutrients associated with that habitat. Nutrients can be derived from the environment or from products of the metabolic pathways of other bacteria within that particular niche (Marsh & Bradshaw, 1997). For example, *Veillonella* spp. are unable to colonise the tooth surface without the presence of *Streptococcus* spp. (McBride *et al*, 1981). *Veillonella* spp. utilise the short chain organic acids produced by *Streptococcus* spp. as an end product of sugar metabolism (Mikx *et al*, 1975). Another example is the stimulation of *T. denticola* growth by the isobutyric acid produced by *P. gingivalis* metabolism (Grenier & Mayrand, 1986).

As dietary nutrients are not always available, members of the stable microbial community cannot rely on these as a constant source of nutrients. Organisms present in the supragingival plaque can derive nutrients from saliva and dietary sources. Saliva itself is sufficient to maintain the growth of major oral species in the absence of dietary nutrients (DeJong *et al*, 1986). The clearance of microorganisms from the oral cavity by swallowing is mediated by saliva as many oral bacteria form large aggregates in the presence of saliva (Koop *et al*, 1989), involving salivary proteins and glycoproteins

(Brady *et al*, 1992; Azen *et al*, 1993; Tabak, 1995). Antibacterial factors such as lysozyme (Golub *et al*, 1985), lactoferrin (Soukka *et al*, 1993), secretory IgA (Liljemark *et al*, 1979), histatins, salivary peroxidases and cystatins are also present in saliva and are thought to have bactericidal, bacteriostatic or inhibitory activity on bacteria in the oral cavity (Rudney, 1995).

The main source of nutrients for subgingival organisms (i.e. in the gingival crevice) is gingival crevicular fluid (GCF), a serum-like fluid that emerges between the surface of the tooth and the epithelial integument (Griffiths, 2003) and flows through the junctional epithelium of the gingivae (Marsh & Martin, 1999). GCF is rich in nutrients needed for the growth of periodontal pathogens such as *P. gingivalis* which require specific growth factors such as hemin and vitamin K1. The composition of GCF is a complex mixture of substances derived from the serum, leukocytes, structural cells of the periodontium and oral bacteria (Uitto, 2003). GCF exudes from the junctional epithelium into the gingival crevice during health or into the periodontal pocket as disease progresses (Lamster, 1997). Sites displaying natural or experimental gingivitis and thus with an increased flow rate of GCF show faster rates of plaque re-growth after removal than sites displaying no inflammation (Hillam & Hull, 1977; Quirynen *et al*, 1991; Ramberg *et al*, 2003) indicating that this nutrient source is beneficial to species present in dental plaque associated with gingivitis.

The flowing action is thought to remove non-adherent cells in the gingival and the flow rate at healthy sites is thought to be in the region of 0.3 μl per tooth per hour (Marsh & Martin, 1999) with the volume range in an undisturbed sulcus being between 0.5-1.0 μl (Lamster *et al*, 1985). This flow rate increases with inflammation, for example, after

orthodontic treatment (Tersin, 1978). After 3 days no brushing a 147% increase from the baseline flow rate of GCF has been observed (Zhang *et al*, 2002) and with periodontitis the flow rate of GCF can increase by up to 30 times (Uitto, 2000). The concentration of specific components of the GCF can be altered during inflammation, for example albumin concentration has been shown to increase during periods of inflammation (Bickel *et al*, 1985). Higher levels of lactoferrin and elastase activity have also been observed around healthy teeth as opposed to those displaying inflammation (Fartash *et al*, 1997).

1.2.1.5 Substratum

The microbial composition of dental plaque sampled from different locations in the mouth has been shown to be distinct due to the variety of surfaces available for colonisation (Aas *et al*, 2005). The teeth provide a solid, non-shedding surface for the accumulation of large numbers of bacteria in the form of dental plaque. The main sites for plaque accumulation are areas which are protected from mechanical shearing forces and the flow of saliva. These include the interdental regions between teeth and the gingival crevice. The species which thrive in these environments vary but are mainly dominated by Gram-positive facultative anaerobes such as streptococci and *Actinomyces* spp. The proteins and glycoproteins present in saliva form the salivary pellicle on the tooth surface which is essential for the attachment of many microorganisms (Clark & Gibbons, 1977; Zahradnik *et al*, 1978; Schilling *et al*, 1992).

Mucosal surfaces such as the gingiva, palate and cheeks are colonised by bacteria to a lower extent due to their shedding nature (Mager *et al*, 2003). The tongue is the most densely populated mucosal surface sustaining the growth of a greater variety of species

1.2.2.1 Protection against environmental challenges

Environmental challenges faced by oral bacteria include exposure to acidit
(Hall-Stoodley *et al*, 2004). Oral bacteria also face competition with othe
the action of host defence mechanisms. Growth as part of a biofilm, pa
presence of the extracellular polysaccharide (EPS) matrix, protects the or
from these challenges. The EPS matrix consists of up to 97% water (Zhan
the exact composition of which varies depending on the available s
microbial composition. Polysaccharides such as hyaluronic acid provi
from desiccation by the retention of water (Sutherland, 2001b). The
provides a physical anchor for oral organisms, protecting them from being
by the shear forces of saliva. Furthermore, the multi-species nature of
allows different organisms to perform functions crucial for the survival of
such as the breakdown of toxic end products of metabolism or by providin
other species by being able to breakdown complex macromolecules (
Haffajee, 2002).

1.2.2.2 Protection against the action of antimicrobial agents

Several mechanisms for the increased resistance of biofilms to antimic
have been discussed by Mah & O'Toole (2001). One of these is
antimicrobial to penetrate the biofilm due to the EPS matrix. The biofilm
may act as a barrier to the diffusion of strongly charged antimicrobial agen

these agents to come out of solution. Fig.1.2.2 demonstrates how the biofilm matrix itself can also act as a barrier for effective penetration of antimicrobial and anti-plaque agents. EPS may act as a protective barrier against the action of antimicrobial agents by preventing access to cells or diluting the concentration of these agents (Stewart, 2002).

Figure 1.2.2: Control of dental biofilms using anti-plaque agents (From Baehni & Takeuchi, 2003). Part (a) of this figure demonstrates when an anti-plaque agent is applied to the tooth surface immediately after cleaning the agent adsorbs to the salivary pellicle and blocks the receptors on the pellicle that would bind to bacterial adhesins thus preventing initial attachment, making this the ideal method of application. Part (b) demonstrates when an anti-plaque agent is applied to a developing biofilm. The agent is effective against bacteria on the surface on the biofilm but not against bacteria attached to the pellicle. Part (c) demonstrates the application of anti-plaque agent to an established biofilm with a significant amount of EPS matrix. The effectiveness of this agent is almost completely lost as the EPS matrix prevents the penetration of the agents into the matrix protecting the bacteria present in the biofilm from any antimicrobial effect.

The diffusion of antimicrobials through biofilms has been reviewed by Stewart (1996). Antimicrobial agents could be neutralized or bound by interacting with components of the EPS matrix, preventing them from reaching the cells in the biofilm. In support of this theory the presence of bacterial biofilms on a surface, as opposed to a biofilm-free surface, has been shown to reduce the diffusion of an antimicrobial agent (Suci *et al*, 1994; Hoyle *et al*, 1992). The prevention of diffusion of an antimicrobial agent by the EPS matrix is due to specific reactions with components of this matrix rather than it simply acting as an impenetrable barrier as some antimicrobials have can move freely through EPS (Gilbert *et al*, 2002). Different antimicrobials display different levels of penetration into the same biofilm (Anderl *et al*, 2000) suggesting that a specific antimicrobial will interact with the EPS matrix in a different way.

Another mechanism of resistance is organisms adopting a slower growth rate when growing as part of a biofilm (Prosser *et al*, 1987; Evans *et al*, 1990a; Evans *et al*, 1990b) which may affect the uptake and thus efficacy of certain antibiotics. Bacteria growing in nutrient limited environments display a slower rate of growth and increased resistance to antimicrobials (Tuomanen *et al*, 1986; Evans *et al*, 1990a; Xu *et al*, 2000; Lewis, 2001) and form persister cells (Lewis *et al*, 2007). Bacteria growing in mature biofilms have been shown to have a reduced rate of growth (Brown *et al*, 1988; Wentland *et al*, 1996). For example, both planktonic and biofilm *Pseudomonas aeruginosa*, growing at a slower rate displayed increased resistance to ciprofloxacin (Evans *et al*, 1991).

It has been suggested that bacteria growing in a biofilm show a reduced rate of growth due to being under a general stress response rather than just nutrient limitation (Brown & Barker, 1999). The stress response regulator RpoS is induced when there is a high density of cells and has been shown to be expressed in *P. aeruginosa* biofilms (Foley *et al*, 1999), whilst *E. coli* lacking RpoS have been shown to be unable to form normal biofilms (Adams & McLean, 1999).Growth as part of a biofilm can also induce the expression of genes for non-specific defence mechanisms such as heat shock proteins and efflux pumps (Socransky & Haffajee, 2002). Due to the heterogeneous nature of the biofilm environment bacteria growing within the same biofilm will be exposed to different environmental conditions and grow at different rates. This has been demonstrated by Wentland *et al*, (1996) where bacteria within the same biofilm showed different relative RNA contents (and thus different growth rates).

Quorum sensing of bacteria growing as part of a biofilm may influence their susceptibility to antimicrobials. *P. aeruginosa* with a mutation in genes involved in quorum sensing formed biofilms with abnormal architecture (Davies *et al*, 1998). A quorum sensing inhibitor was able to prevent the formation of *Staphylococcus epidermidis* biofilms (Balaban *et al*, 2003).

Cells growing as part of a biofilm may express a biofilm phenotype making them more resistant to the action of antimicrobials. Mah *et al*, (2003) found that cells in *P. aeruginosa* biofilms express a gene for the production of glucans that interact with specific antimicrobials thus making these communities resistant. Multidrug efflux pumps have also been show maximal expression at the biofilm-substratum interface (De Kievit *et al*, 2001).

1.3. Periodontal diseases and the oral microbiota

The resident microbiota at a particular site in the mouth will remain relatively stable over time due to the microbial homeostasis achieved by the dynamic microbial interactions between oral species that prevent minor fluctuations in the oral environment from disrupting these populations (Marsh, 2003b). Significant changes in environmental factors may result in the development of periodontal diseases (Marsh & Bradshaw, 1997). The changes that occur in the oral microbiota with the development of gingivitis are due to bacterial succession. When adequate oral hygiene measures are in place the stable or perhaps arrested community that develops is different to the population that will develop when hygiene measures are ceased and the composition of the oral community is allowed to evolve further. The limitation of succession in healthy plaque is physical; species are occupying the environmental niche that could be used by disease causing species and are better able to survive in this environment.

1.3.1 Plaque hypotheses

The non-specific plaque hypothesis proposes that periodontal disease results from large accumulations of plaque producing large amounts of noxious products, stimulating host defenses and thus causing periodontal disease. This concept dates as far back as the 19th century (Miller, 1890 cited by Loesche & Grossman, 2001) before there was any concept of the natural microbial diversity in the oral cavity. According to this hypothesis identifying specific organisms as agents in disease is not important as the entire bacterial mass is thought to be responsible for the resulting disease. The specific plaque hypothesis (Loesche, 1976) proposes that plaque with elevated proportions of specific pathogenic organisms which stimulate a host response would result in disease. The ecological plaque hypothesis (Marsh, 1991) reconciles some of the difference

between the specific and non-specific hypotheses. The ecological plaque hypothesis proposes that environmental factors are driving the changes in the resident oral microbiota, namely the inflammatory response instigated by the accumulation of dental plaque at gingival margins and changes in the redox potential (Marsh & Martin, 1999). Species which were only minor members of the community are encouraged to proliferate under these new environmental conditions and become more significant members of the community. These species are selected for by the reduced redox potential which encourages the growth of obligate anaerobes and the increased availability of GCF encouraging the growth of proteolytic species.

Figure 1.3.1: The Ecological Plaque Hypothesis.

(From Marsh, 1994 G + represents Gram-positive bacteria; G – represents Gram-negative bacteria).

1.3.2 Gingivitis

Fig. 1.3 2 illustrates periodontal health and the development of the periodontal diseases gingivitis and periodontitis. Accumulation of dental plaque at gingival margins due to inadequate dental hygiene leads to the inflammation (a non-specific inflammatory) response of the gingiva, defined as gingivitis (Loe *et al*, 1965), one of the most common diseases of humans, affecting most adults at some point in their lives. The most common form of gingivitis is chronic marginal gingivitis or plaque-related gingivitis. This is a reversible condition as a return to meticulous dental hygiene practices will restore gingival health (Page, 1986). As the gingiva become more inflamed the gingival sulcus deepens (Listgarten, 1994). This leads to increased subgingival plaque as the space in which plaque can accumulate and the availability of nutrients, in the form of gingival crevicular fluid (GCF), in this environment is increased. Children are more resistant to the development of gingivitis, displaying different periodontal microbiotas to adults (Moore *et al*, 1984). Gingivitis becomes more prevalent during puberty and early adulthood due to increased hormone availability encouraging the growth of species associated with gingivitis (Mariotti, 1999).

Other less common forms are linked to specific circumstances including vitamin deficiency (Nishida *et al*, 2000), infections such as HIV (Winkler & Robertson, 1992), pregnancy gingivitis (Raber-Durlacher *et al*, 1994) which is associated with increased hormone levels (Zachariasen, 1989) and leukemia (Willershausen *et al*, 1998). Acute necrotising ulcerative gingivitis (ANUG) involves the formation of a grey pseudomembrane on the gingivae which sloughs off to reveal a bloody necrosis (Rowland, 1999) and is often linked to smoking and poor oral hygiene (Johnson & Engel, 1986). Reduced immunity and emotional and physiological stresses have also

been linked to the development of ANUG (Breivik *et al*, 1996). It typically affects people under the age of 35 (Stevens *et al*, 1984) and occurrence is rare. It is a much more serious condition than other forms of gingivitis and has a much more defined microbial aetiology involving high levels of *Treponema* spp., *Selenomonas* spp., fusiforms and *Prevotella* spp. (Loesche *et al*, 1982). Microbial cells are seen to invade host tissues (Dahle *et al*, 1993) causing much of the tissue destruction associated with this condition.

Figure 1.3.2: Periodontal disease development.

(Adapted from www.perio.org).

1.3.2.1 The microbiota of gingivitis

Identifying species related to disease is not a simple case of detecting species which are not usually associated with the dental plaque of health. Many species which are implicated in gingivitis are present in healthy dental plaque. The subgingival plaque community is richer in species than the supragingival plaque community, although

many subgingival species can be isolated from supragingival plaque (Gmur & Guggenheim, 1994).

Proportional increases in a range of bacterial species are implicated in the etiology of gingivitis. The microbial population associated with gingivitis represents the bacterial succession that has occurred in these communities. The species associated with periodontal disease are summarised in the Table 1.3.2.

Table 1.3.2: Predominant bacteria of gingivitis in young adult humans (Marsh & Martin, 1999).

Early studies revealed the predominant cultivable microorganisms inhabiting gingival crevices affected with a chronic gingivitis consisted of 29.1% rods which were mainly *A. naeslundii*, *A. israelii*, and *A. viscosus* (Slots *et al*, 1978). *S. mitis* and *S. sanguinis*

together made up a significant portion (26.8%) of the cultivable organisms. Gram-negative anaerobic rods constituted 25% of the total isolates with *F. nucleatum*, *Streptococcus intermedius*, *Bacteroides melaninogenicus, B. ochraceus*, other *Bacteroides* spp., *Selenomonas sputigena*, and *Campylobacter sputorum* as the most predominant isolates. *Haemophilus parainfluenzae* averaged about 14% and *Veillonella* species 4.3% of the cultivable microbiota. Fusiform bacteria (including *Fusobacterium* spp., *Leptotrichia buccalis*, *T. forsythia* and *Capnocytophaga* spp.) display high prevalence in gingival margin plaque from patients with gingivitis (Gmur *et al*, 2004), *Capnocytophaga* spp. also show increased proportions in patients displaying rapid plaque formation compared to slow plaque formers (Zee *et al*, 1996). The increased prevalence of *Capnocytophaga* spp. is indicative of the plaque environment becoming less aerobic as a result of plaque accumulation.

Periodontal disease development seems to be a subject-specific process as a common finding from experimental gingivitis studies is that there is great diversity in the composition of the oral microbiota between individuals (Moore *et al*, 1984). The composition of the microbiota may be a key determinant of the type and severity of periodontal disease that develops. The DGGE profiles of subgingival plaque samples from different subjects with gingivitis showed profiles with different banding patterns in terms of migration and band intensity but were similar in terms of species richness and diversity (Gafan *et al*, 2005).

1.3.2.1.1 *Actinomyces* spp.

From experimental gingivitis studies *Actinomyces* spp. have been found to increase in numbers as gingivitis develops (Loesche & Syed 1978; Moore *et al*,1984; Tanner *et al*, 1996) and are associated with mature plaque (Ellen, 1976; Slots, 1978; Syed & Loesche,

1978). *A. naeslundii* was found to be one of dominant cultivable organisms from patients with gingivitis (Tanner *et al*, 1998) and has been associated with active periodontal patients (Tanner *et al*, 1996), *A. israelii* has specifically been linked with developing gingivitis (Moore, 1982) while *A. viscosus* has been linked to bleeding gingivitis (Loesche & Syed, 1978) and puberty gingivitis (Mombelli *et al*, 1990). *A. israelii* and *A. naeslundii* were also identified as major isolates from the subgingival microbiota of puberty gingivitis patients (Tsuruda *et al*, 1989).

This increase in *Actinomyces* spp. may be involved in the progression of periodontal disease as *A. israelii*, *A. naeslundii* and *A. viscosus* have all been shown to stimulate lymphocyte transformation (Baker *et al*, 1976) which is correlated with increased severity of periodontal disease. *Actinomyces* species have been shown to attach to buccal epithelial cells (Saunders & Miller, 1980) and gingival epithelial cells. Enzymes present in gingival crevicular fluid have been shown to significantly enhance the ability of *A. viscosus*, *A. naeslundii* and *A. israelii* to attach to epithelial cells while the ability of oral streptococci to attach was significantly reduced (Childs & Gibbons, 1990). They can also be involved in the attachment of periodontal pathogens. For example, *A. viscosus* has been shown to adhere to *P. gingivalis* on surfaces mimicking the tooth surface (Slots & Gibbons, 1978; Li *et al*, 1991; Takazoe *et al*, 1984).

1.3.2.1.2 Fusobacteria

A. naeslundii and *F. nucleatum* have been shown to be the most commonly isolated species from the human gingival crevice (Moore & Moore, 1994). In this study *F. nucleatum* was also found to increase significantly in sites with a gingival index score of 1. As butyric acid is the major end product of the metabolism of *F. nucleatum*, increased

levels of this organism are thought to be a cause of initial gingival irritation (Slots, 1977). As mentioned earlier, the increased presence of *F. nucleatum* may serve as a mechanism for attachment of periodontal pathogens such as *P. gingivalis*.

1.3.2.1.3 Black pigmenting anaerobes (BPAs)

BPAs are obligate anaerobes and produce black pigment which protects the cells from oxygen which is toxic. These include species of the genus *Porphyromonas* and some *Prevotella* (*Pr. intermedia*, *Pr. nigrescens*, *Pr. melaninogenica* and *Pr. loescheii*). Black-pigmenting anaerobes are detected at low frequencies in the dental plaque associated with health (Zambon *et al*, 1981) but have been shown to be more frequently isolated in higher proportions from sites with gingivitis (White & Mayrand, 1981). Increasing levels of *Prevotella* spp. are associated with increasing gingivitis scores (Loesche & Syed, 1978). *P. gingivalis* is also more frequently associated with subgingival samples from patients with gingivitis but is more strongly associated with periodontitis (Ashimoto *et al*, 1996) which will be discussed further in the next section.

1.3.2.1.4 Spirochaetes

Spirochaetes can be members of the microbial community of the supragingival plaque of individuals with healthy gingiva (Barron *et al*, 1991) but are more commonly associated with the subgingival plaque which is predominantly composed of Gram-negative organisms. The presence of specific bacteria in healthy dental plaque may predispose certain individuals to gingivitis (Riviere & DeRouen, 1998). The presence of *T. denticola* in health-associated dental plaque correlated with future development of gingivitis. *Treponema socranskii* linked to increasing gingival index scores (Moore *et al*, 1984). Four species of oral spirochaetes have been consistently identified (Chan &

McLaughlin, 2000), *T. denticola*, *Treponema pectinovorum*, *T. socranskii* and *Treponema vincentii*. These species are thought to play an important role in periodontal disease due to the virulence factors they possess (Fenno & McBride, 1998) and their ability to pass through viscous environments such as gingival crevicular fluid and to penetrate epithelial cells and gingival connective tissue.

1.3.2.1.5 Other Gram-negative species associated with gingivitis

Capnocytophaga spp. are seen to increase during experimental gingivitis (Moore *et al*, 1982; Moore *et al*, 1987; Tanner *et al*, 1998; Gmur *et al*, 2004) as conditions become more favourable for their growth but do not appear to account for a significant proportion of the total cultivable microorganisms. They have also been shown to be linked to puberty gingivitis (Mombelli *et al*, 1990). There are at least seven *Campylobacter* spp. which have been isolated from subgingival plaque (Machuch & Tanner, 2000) with *C. rectus* (formerly *Wolinella recta*) implicated as a periodontal pathogen (Tanner & Bouldin, 1989; Tanner *et al*, 1998). *Campylobacter* spp. have been shown to be associated with periodontal diseases (Slots *et al*, 1978; Tanner *et al*, 1979, Wade *et al*, 1991). *E. corrodens* is a putative periodontal pathogen and has been shown to increase during experimental gingivitis (Moore *et al*, 1987; Tsuruda *et al*, 1995).

1.3.2.1.6 Other Gram-positive species associated with gingivitis

Gram-positive species associated with gingivitis include *Eubacterium* spp. (Moore *et al*, 1982; Moore *et al*, 1987), *P. micros* (Slots *et al*, 1978; Kremer *et al*, 2000) and *Lactobacillus* spp. (Moore *et al*, 1982; Moore *et al*, 1987).

1.3.2.1.7 Uncultivable species and gingivitis

The uncultivable portion of the oral microbiota associated specifically with gingivitis has not been extensively examined. Kroes *et al*, (1999) assessed the range of species present in a subgingival plaque sample from a single patient with mild gingivitis using PCR and cloning of the 16S rRNA region. Of the 264 sequences examined, 48% did not match significantly with any species compared to 21% of the cultivated species that were compared to the 16S rRNA database. The diversity of species present in subgingival samples from gingivitis and no gingivitis patients was examined by Gafan *et al*, (2005a) using DGGE. They found that there was a greater biological diversity in the sample group with no gingivitis than the gingivitis group inferring that a decrease in bacterial diversity may be associated with the shift from health to gingivitis and that the dental plaque community associated with health is more stable than that associated with gingivitis. They suggested this apparent decrease in diversity is a result of certain taxa proliferating as a consequence of inflammation masking the presence of taxa that are still present but do not necessarily increase in number. They also observed some bands that were significantly associated with gingivitis.

1.3.3 Periodontitis

Periodontitis, a more severe form of periodontal disease, is less prevalent than gingivitis, occurring in approximately 35% of adults in the US (Albandar *et al*, 1999) and being more common in individuals over the age of 30. Periodontitis is defined as inflammation of the periodontium which leads to progressive bone loss around the teeth (Fig. 1.3.2) which can ultimately lead to tooth loss. Pre-existing gingivitis is often a risk factor in the development of periodontitis. The prevention of gingivitis is therefore thought to be a primary preventative measure for periodontitis (Kinane, 2001), but other

factors are involved such as tobacco use (Haber *et al*, 1993; Tonetti *et al*, 1998; Johnson & Slach, 2001), diabetes (Oliver & Tervonen, 1994; Grossi & Genco, 1998), genetic susceptibility (Hart & Kornman, 1997) and hormonal changes, for example associated with pregnancy (Offenbacher *et al*, 1998). The types of periodontitis were reclassified at the 1st European Workshop in Periodontotolgy (Armitage, 1999) to give two defined types.

1.3.3.1 Chronic Periodontitis

Chronic periodontitis is the most common form of periodontitis. The accumulation of plaque leads to loss of clinical attachment including destruction of the periodontal ligament and the adjacent supporting bone (Kumar *et al*, 2003). This form of disease is seen mostly in adults and is the most common cause of tooth loss above the age of 30. The amount of destruction is thought to be directly linked to the amount of plaque accumulation. There is progressive tissue destruction in discontinuous episodes (Moore *et al*, 1982).

1.3.3.2 Aggressive Periodontitis

Aggressive periodontitis was previously described as early onset periodontitis as it is most common in individuals under the age of 35. There is significant attachment loss in the absence of other local factors such as plaque or calculus. The development of this form of periodontitis is linked to immune deficiencies. In the juvenile form there is severe bone loss around the 1st molars and incisors with the absence of inflammation, bleeding and heavy plaque accumulation. In the rapidly progressive form that affects individuals from the early 20s to mid 30s severe inflammation is observed along with rapid bone loss.

1.3.3.3 Microbiology

The microbial challenges associated with periodontitis induce an immediate inflammatory and immune response, the nature and magnitude of this response is influential on the severity and rate of progression of periodontitis (Page & Kornman, 1997). Ashimoto *et al*, (1996) proposed that *A. actinomycetemcomitans*, *T. forsythia*, *C. rectus*, *E. corrodens*, *P. gingivalis*, *Pr. intermedia*, *Pr. nigrescens* and *T. denticola* were all periodontal pathogens as they were all isolated at increased frequency in subgingival samples from patients with periodontitis. Tanner *et al*, (1996) found that species associated with putative active periodontitis included *A. naeslundii*, *V. parvula*, *Selenomonas noxia* and *Pr. nigrescens*. *S. sanguinis*, *S. gordonii* and *Peptostreptococcus micros* were associated with inactive subjects. *S. gordonii* and *S. oralis* were associated with health, whereas *Pr.nigrescens* was associated with gingivitis. Elevated serum antibodies were detected to *A. actinomycetemcomitans* in 4 subjects. The predominant microbiota of putative active subjects included some species previously associated with gingivitis, and some species previously associated with progressing periodontal disease.

Species more strongly associated with chronic periodontitis include *P. gingivalis*, *Pr. intermedia* and *C. rectus* (Slots, 1977; Dzink *et al*, 1988). Species more strongly associated with aggressive periodontitis include *A. actinomycetemcomitans*, *Capnocytophaga* spp. and *E. corrodens* (Slots *et al*, 1976; Muller & Flores-de-Jacoby, 1985; Delaney & Kornman, 1987). Gram-positive species associated with periodontitis include *P. micros* (Slots *et al*, 1977; Rams *et al*, 1992; Moore *et al*, 1991; Haffajee & Socransky, 1994), the cell wall of which is found to induce a pro-inflammatory response in human macrophages (Tanabe *et al*, 2007) and *Eubacterium* spp. (Slots *et al*, 1977;

Uematsu & Hoshino, 1992) for which a diverse range of species have been detected in subgingival samples of patients with periodontitis.

Periodontopathic bacteria have also been identified in the gingival tissues of Japanese patients (Thiha *et al*, 2007). *P. gingivalis*, *T. forsythia* and *A. actinomycetemcomitans* were all localised in diseased gingival tissues and thus implicated in periodontal tissue destruction whereas species such as *S. sobrinus* and *S. oralis* which are not implicated as periodontal pathogens were detected at low levels in these tissues.

P. gingivalis, *T. denticola*, and *T. forsythia* are said to form a pathogenic consortium in periodontitis (Holt & Ebersole, 2005), the presence of which is thought to be a major etiologic contributor to common adult forms of periodontitis (Socransky *et al*., 1998).

1.3.3.3.1 *Porphyromonas gingivalis*

P. gingivalis is strongly associated with individuals with advanced and severe periodontal disease (Ashimoto *et al*, 1996). A number of these organisms can invade gingival epithelial cells and are able to replicate within these cells (Lamont *et al*, 1995) and thus have a significant role in the pathology of periodontal diseases. *P. gingivalis* also produce a variety of virulence factors including active proteases, haemolysin, collagen degrading enzymes, bone resorption inducing toxin (Mihara *et al*, 1993) and are also able to adhere to oral epithelial cells via extracellular components such as fimbriae (Meyer & Fives-Taylor, 1993) which mediate their adherence to oral epithelial cells and also are able to invade these cells (Lamont *et al*, 1995). *P. gingivalis* also adhere to other bacteria including *F. nucleatum* (Kinder & Holt, 1993), *T. denticola* (Grenier, 1992b) and *T. medium* (Umemoto *et al*, 1997).

1.3.3.3.2 *Tannerella forsythia*

T. forsythia has been implicated as a periodontal pathogen (Grossi *et al*, 1994; 1995; Tanner *et al*, 1998) and possesses several virulence factors that may be involved in this role. Inagaki *et al*, (2005) examined the regulation of the bspA gene (a surface associated secreted protein) during biofilm growth and contact stimuli (interbacterial interactions). The levels of bspA transcript were found to be significantly reduced as a result of contact stimulus and in biofilm cells relative to planktonic cells. They suggest that the down-regulation of the BspA protein in biofilms may have implications in pathogenesis as a plausible mechanism of evasion of host immune responses. Other virulence factors include proteases (Saito *et al*, 1997), sialidases (Braham and Moncla, 1992; Ishikura *et al*, 2003), an apoptosis-inducing activity (Arakawa *et al*, 2000) and hemagglutinin (Murakami *et al*, 2002). A product or a component of *T. forsythia* seemed to stimulate growth of *P. gingivalis* under nutrition-limited conditions (Yoneda *et al*, 2005) and they suggested gingipains played an important role in digestion or uptake of this growth-promoting factor and the interaction between *T. forsythia* and *P. gingivalis* in growth may be in part related with synergistic virulence.

1.3.3.3.3 *Treponema denticola*

Oral treponemes have been isolated in increasing numbers from patients with periodontal lesions (Fiehn, 1989; Penn, 1989). *T. denticola* has been quantitatively linked to severe periodontitis (Simonson *et al*, 1988) and is associated with the severity of periodontal tissue destruction (Takeuchi *et al*, 2001). Sectioning of subgingival plaque samples has shown these organisms to be more prevalent in surface layers of subgingival plaque with *P. gingivalis* being more dominant in deeper layers (Kigure *et al*, 1995). The accumulation of *T. denticola* and its products in the periodontal pocket

may damage the surface lining periodontal cells (Uitto *et al*, 1995).The virulence factors of oral treponemes are reviewed by Fenno & McBride (1998) and include outer-sheath-associated peptidases, proteinases, adhesins that bind to matrix proteins and cells, and an outer-sheath protein with pore-forming properties. The clinical data regarding the presence of *T. denticola* in periodontal health and disease, together with the basic research results involving the role of *T. denticola* factors and products in relation to periodontal diseases have been reviewed and discussed by Sela (2001).

1.3.3.3.4 *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans has been implicated in aggressive periodontitis (Ebersole *et al*, 1982; Zambon *et al*, 1983; Novak & Novak, 1996) and demonstrate a number of virulence factors which may be involved in their role as a periodontal pathogen. These include the production of leukotoxin (Tsai *et al*, 1984), collagenase, immunosuppressive factors and invasins. The lipopolysaccharide of *A. actinomycetemcomitans* is also induces a potent cytokine response (Fives-Taylor *et al*, 1999).

1.3.3.3.5 Uncultivable bacteria and periodontitis

The uncultivable portion of the microbial community has been investigated much more thoroughly for periodontitis than for gingivitis. Many studies have examined the richness and diversity of species present (Tanner *et al*, 1998; Ximenez-Fyvie *et al*, 2000a; 2000b; Paster *et al*, 2001; Hutter *et al*, 2003; Haffajee *et al*, 2005; Sakamoto *et al*, 2004; Kumar *et al*, 2005; Paster *et al*, 2006; Tanner *et al*, 2006) and from examining this portion of the community it has become apparent that uncultivable bacteria could have a key role in the development of periodontitis. Kumar *et al*, (2005) examined the microbiota associated with periodontitis. They found *Peptostreptococcus* and *Filifactor*

spp. were the most numerous taxa identified. *Megasphaera* and *Desulfobus* were also found to be associated with periodontitis. Newly identified phyla and named species of *Campylobacter*, *Selenomonas*, *Deferribacteres*, *Dialister*, *Catonella*, *Tannerella*, *Streptococcus*, *Atopobium*, *Eubacterium* and *Treponema* spp. all showed increased levels associated with periodontitis and interestingly these candidates far outnumbered *P. gingivalis*. *Streptococcus* and *Veillonella* spp. were generally associated with healthy subjects.

The association of *Archaea* and chronic periodontitis was investigated by Lepp *et al*, (2004) using quantitative PCR, and increasing levels of archael DNA were found to be associated with increasing severity of disease, with rDNA phylotypes from the genus *Methanobrevibacter* being most commonly identified. Archael DNA was found in 36% of chronic periodontitis subjects and interestingly was not detected at all from healthy subjects.

1.3.4 Peri-implantitis

Peri-implantitis, the inflammation of the soft tissues around a dental implant is also associated with the accumulation of dental plaque. Dental implants can serve as an ideal surface for oral biofilm formation, particularly if they have a rough surface. This plaque accumulation on the implant instigates the host inflammatory response in the same way that plaque accumulation on the tooth surface leads to gingivitis and periodontitis (Berglundh *et al*, 1992) making the microbial aetiology of peri-implantitis similar to that observed with gingivitis (Meffert, 1996; Leonhardt *et al*, 2003). In experimental studies where patients were asked to refrain from oral hygiene for periods up to 3 weeks, inflammation developed in a similar way as observed with experimental

gingivitis (Pontoriero *et al*, 1994). Differences in microbiota have been observed with successful and failed implants (Rams *et al*, 1984; Becker *et al*, 1990; George *et al*, 1994), with peri-implantitis patients harbouring high levels of periodontal pathogens (Hultin *et al*, 2002). This inflammation around the implant can ultimately lead to failure of the implant, having a significant impact on dental costs and patient discomfort. To minimize this risk the use of antimicrobial agents after dental surgery is essential. Due to the similar nature of the development of peri-implantitis and periodontal diseases treatments for periodontal disease are thought to be effective against the development of peri-implantitis (Mombelli & Lang, 1998).

1.4 Preventing periodontal diseases

The importance of maintenance of stable microbial populations in the development of periodontal diseases has been highlighted in a study by Kumar *et al*, (2006). The group monitored the composition of the microbiota of the gingival sulcus over a two year period in relation to periodontal status in 25 subjects. The microbial composition was assessed by cloning and sequencing of the16S rDNA amplicons. They observed that subjects, whose periodontal status worsened over the 2 year period, showed greatest instability in their bacterial composition, providing evidence that changes in periodontal status are associated with shifts in the bacterial community. In subjects that remained periodontally healthy, on average 75% of species were maintained, whilst for subjects whose periodontal status worsened less than half of species were maintained. A larger range of species was also observed in these subjects. Means by which these stable populations can be maintained include mechanical plaque removal and the use of anti-plaque agents.

1.4.1 Mechanical plaque removal

The ideal method of plaque control is mechanical removal, both professional cleaning and day to day measures such as the use of mouthwash and electric toothbrushes (Needleman *et al*, 2005). Meticulous dental hygiene combined with interdental cleaning has been shown to prevent gingivitis (Axelsson *et al*, 1994). Studies on the individual dental habits of adults in industrialised countries have shown that the average time spent for mechanical plaque removal is not adequate to ensure the complete removal of interdental plaque, which is often the most frequent site of gingival inflammation (Baehni & Takeuchi, 2003). Therefore, it has been suggested that including anti-plaque agents in regularly used dental products (toothpastes and mouthwashes) may reduce

gingivitis in the general population which in turn could have a major impact on costs for periodontal care (Baehni & Takeuchi, 2003).

The effect of supragingival plaque removal on the composition of the subgingival microbiota has been investigated in several studies (Haffajee *et al*, 2001; Hellstrom *et al*, 1996; Westfelt, 1998; Ximenez-Fyvie *et al*, 2000c; Al-Yahfoufi *et al*, 1995). It was observed that repeated professional removal of supragingival plaque influenced the composition of the subgingival microbiota, reducing total bacterial counts as well as number of species thought to be involved in periodontal disease. The supragingival plaque may be a source of pathogenic microorganisms that can thrive in the subgingival environment (Smulow *et al*, 1983; Quirynen *et al*, 2005) and may also be a source of nutrients for the subgingival plaque (Haffajee *et al*, 2001). One hypothesis for this change in subgingival plaque composition is that removal of supragingival plaque encourages a "change in habitat" (Ximenez-Fyvie *et al*, 2000c) with decreased inflammation and gingival crevicular fluid flow, resulting in lower levels of nutrients for subgingival organisms (Daly & Highfield, 1996; Ramberg *et al*, 1996).

The prevention of plaque accumulation using mechanical or chemical techniques is the ideal regime to prevent the development of destructive periodontal diseases (Baehni & Takeuchi, 2003). The effectiveness of this approach was evaluated by Teles *et al*, (2007). Using checkerboard DNA-DNA hybridisation they examined the microbial composition of subgingival plaque samples over a 3 year period. Subjects received dental prophylaxis and professional plaque removal every six months during the study as well as employing a specific dental regime at home. Total counts of bacteria were reduced over the three years, major reductions occurring by the second year for

members of the genera *Actinomyces*, *Capnocytophaga*, *Campylobacter*, *Fusobacterium* and *Prevotella*, all genera which have been implicated in periodontal diseases.

1.4.2 Chemical control of plaque

1.4.2.1 Difficulties in targeting biofilms with chemical agents

The increased resistance of organisms to antibiotics when growing as part of a biofilm as opposed to growing in planktonic culture is well known and documented (Evans *et al*, 1990b; Duguid *et al*, 1992; Ashby *et al*, 1994; Aaron *et al*, 2002) and has been discussed in Section 1.2.2.2.

1.4.2.2 Agents used for the control of supragingival plaque

Due to the difficulties of targeting well established biofilms for treatment as mentioned earlier, an effective approach is to prevent the accumulation of dental plaque rather than treatments to eradicate plaque once it is formed. Antimicrobial agents are commonly delivered to the oral cavity in the form of toothpastes and mouthwashes. They are initially present in high concentrations but are quickly washed away by saliva flow.

Effective inhibition of *in vitro* plaque formation requires drug substantivity (adsorption and retention by the oral environment) and solubility in the oral environment in order to be effective. Agents that have been assessed for these properties include chlorhexidine and tetracycline. The aim with such agents is to reduce the total bacterial load, not complete eradication of dental plaque by maintaining the levels of species associated with health.

1.4.2.2.1 Chlorhexidine

Chlorhexidine is considered to be the gold standard in the control of supragingival plaque and has been used extensively for the prevention of dental plaque accumulation. It is a broad spectrum antiseptic which has been shown to act against Gram-positive and Gram-negative organisms, aerobic and anaerobic organisms by *in vitro* studies (Hennessey, 1983; Emilson, 1977). Chlorhexidine has been shown to have good substantivity in the oral environment, adsorbing and binding to soft and hard tissues in the mouth and thus is maintained at functional concentrations for sustained lengths of time. In experimental gingivitis studies where subjects were given 0.2% chlorhexidine digluconate mouthwash before stopping oral hygiene measures, less severe symptoms of gingivitis were observed (Loe *et al*, 1971; Addy 1996; Hase, *et al*, 1995; Hull, 1980). Chlorhexidine is only effective against the build up of plaque and will not work against plaque which is already accumulated as it prevents the initial attachment of bacteria by adsorbing to the surface of the tooth and by binding to the bacterial membrane (Eley, 1999).

The effect of chlorhexidine against oral bacteria when grown as a biofilm as opposed to planktonic cells is significantly reduced (Wilson *et al*, 1996). Vitkov *et al*, (2005a) observed the ultrastructural alterations caused by exposure to 0.1% chlorhexidine on *ex-vivo* oral biofilms using electron microscopy. The effect was loss of bacterial membrane integrity and fimbrial disintegration as had been observed with planktonic cells but in biofilms this was restricted to the outermost layers, accounting for only a very small proportion of the biofilms being affected. Studies of the effects of chlorhexidine pulsing on *in vitro* oral biofilms (Pratten *et al*, 1998a; b) have shown that after an initial decrease in bacterial numbers immediately after pulsing numbers rapidly returned to

levels seen before pulsing. After repeated pulsing, no effect was seen even immediately after pulsing, highlighting the ineffectiveness of this agent for prolonged use against the accumulation of dental plaque. This effect was seen on both single species and microcosm biofilms. However, when chlorhexidine was applied to the substratum before exposure to bacteria then subsequently pulsed in the bacterial viability of microcosm biofilms was reduced. Chlorhexidine diacetate varnishes applied to the dentition have been shown to prevent the accumulation of plaque, *A. naeslundii* and *A. viscosus* levels were suppressed for two weeks after treatment and *S. mutans* levels were reduced for up to four weeks (Schaeken & De Haan, 1989). Chlorhexidine has been incorporated into dental materials (Palmer *et al*, 2004; Takahashi *et al*, 2006) showing sustained release and antibacterial activity. This antibacterial effect of incorporated chlorhexidine has been demonstrated against planktonic oral species (Ribeiro & Ericson, 1991) and against oral biofilms (Leung *et al*, 2005).

One problem with chlorhexidine use is the staining of teeth, usually caused by the combination of chlorhexidine with dietary components to produce brown staining. Lang *et al*, (1998) observed that of several anti-plaque agents tested chlorhexidine digluconate was the most effective agent used against the accumulation of plaque and gingivitis but was found to be the least preferred method by patients. Increased supragingival calculus formation can also occur (Yates *et al*, 1993). Prolonged use of this agent is not recommended as it is best used as a preventative measure against plaque formation during periods where mechanical methods of plaque removal are not possible.

1.4.2.2.2 Tetracycline

Tetracycline and its derivatives strongly bind to the tooth surface and is released in its active form from this surface (Baker *et al*, 1983). It also adsorbs to dentally relevant materials such as hydroxyapatite (Misra, 1991). Tetracycline has been found to adsorb to saliva-coated enamel and to inhibit *in vitro* plaque formation by pure cultures of oral bacteria including *A. viscosus*, *A. naeslundii* and *S. mutans* (Baker *et al*, 1983). When applied topically its antibacterial effect can be limited against certain species. *Pr. intermedia* biofilms were shown to be unaffected by topical application of tetracycline in increasing concentrations (Takahashi *et al*, 2006), interestingly at some concentrations biofilm formation increased. Tetracycline was shown to significantly alter the microbial composition of microcosm biofilms developed in the CDFF (Ready *et al*, 2002) when pulsed onto already established biofilms. Total bacterial counts did not return to levels seen before pulsing and increased proportions of tetracycline resistant bacteria were observed due to tetracycline sensitive bacteria being reduced in the population. Controlled release of tetracycline into periodontal pockets of patients with periodontitis has been achieved with tetracycline releasing fibres (Goodson *et al*, 1983) and injectable gels (Maze *et al*, 1996) and has been shown to reduce periodontal pathogens immediately after application (Lowenguth *et al*, 1995).

1.4.2.2.3 Metal compounds

Zinc (Afseth *et al*, 1983), stannous (Attramadal & Svatun, 1984) and copper (Afseth *et al*, 1983) have all been shown to be retained in the oral cavity for reasonable amounts of time after use in mouthwashes and toothpastes. Sites of retention are thought to be the oral mucosa, tooth pellicle and supragingival plaque (Cummins & Creeth, 1992).

1.4.2.2.3.1 Silver ions

Several studies have shown that silver ions can have toxic effects against prokaryotic organisms while having no effect against eukaryotic cells (Marino *et al*, 1974; Berger *et al*, 1976) making them an ideal candidate for use as a selective agent against bacteria without damaging host tissues for the treatment of periodontal diseases. Yamamoto *et al*, (1996) found that composite resin containing silver ions was effective at reducing bacterial numbers when incubated with planktonic cultures of three oral species of streptococci. Kawahara *et al*, (2000) tested the antibacterial effect of silver zeolite against several oral bacteria and found it to be more effective against Gram-negative bacteria such as *P. gingivalis*, *A. actinomycetemcomitans* and *Pr. intermedia* than Gram-positive species such as *S. mutans*, *S. sanguinis* and *A. viscosus*. Similar observations were made by Spacciapoli *et al*, (2001) where silver nitrate was found to be much more effective at killing periodontal pathogens including *P. gingivalis*, *Pr. intermedia*, *Prevotella denticola*, *T. forsythia*, *F. nucleatum* subspecies *vincentii*, *Campylobacter gracilis*, *C. rectus*, *E. corrodens*, and *A. actinomycetemcomitans* than oral streptococci. As Gram-negative species are more commonly isolated from dental plaque samples as gingivitis develops, agents that are more effective against these species would be useful in maintaining microbial populations associated with health.

Mulligan *et al*, (2003) tested the effect of phosphate based glasses containing silver ions on the development of *S. sanguinis* biofilms and found that bacterial counts were significantly reduced for the first 48 hours of biofilm development. They proposed that the initial decrease in growth rate was due to the release of silver ions but once a significant layer of bacteria had developed on the surface of the glass this silver ion release would not be reaching the layer of viable bacteria and thus normal biofilm

growth could commence. Ahmed *et al*, (2006) similarly observed that phosphate based glasses doped with silver showed significant antibacterial effect against planktonic *S. aureus*, *E. coli*, and *Candida albicans*, silver ions showing prolonged release suggesting that the reduced effect of silver ions against biofilm development is not due to silver ions no longer being released but due to the silver ions being less effective against developed biofilms, possibly due to decreased penetrance into the biofilm matrix or increased antibacterial resistance of bacteria in biofilms.

1.5 Analysis of dental plaque

1.5.1 Characterisation of the microbial population

Many techniques have been employed to characterise the microbial composition of dental plaque communities. The strengths and weaknesses of each approach are summarised in Table 1.2.1.

Method	Strengths	Weaknesses	Applications
Non- selective media	Can detect unrecognised species; provides cultures for further analysis.	Extremely time consuming; often difficult to speciate cultures. Cannot provide all combinations of specific nutrients etc to grow a large proportion of the microbiota.	Studies of new ecosystems.
Selective media	Modest numbers of samples for modest number of species.	Few useful selective media available; often too selective or not selective enough; expensive.	Studies of limited scope involving 1-10 species in modest number of samples.
Immunofluorescence	Specificity; reasonably rapid.	Limited number of useful antisera; small numbers of samples may be run.	May be more useful for diagnostic rather than ecologic or treatment studies.
Fluorescent in situ hybridisation (FISH)	Specificity, used for uncultivable species.	Expensive, limited number of species can be visualised at once.	Spatial location of species within a biofilm.
PCR	Sensitivity; specificity.	Not quantitative (presence/absence); expensive; dependent on amplification.	Detection of species in a subject (prevalence); detection of low numbers of species post therapy.

Table 1.5.1: Methods to determine microbial composition of oral samples.

(From Socransky & Haffajee, 2005)

1.5.1.1 Culture dependent techniques

The use of traditional culture techniques has been used extensively to characterise the microbial populations of dental plaque, specifically to detect changes in microbial populations associated with periodontal diseases (Syed & Loesche 1978; Loesche & Syed 1978; Moore *et al*, 1982; 1984; Moore & Moore, 1994; Mombelli *et al*, 1990). Initial assessment of the microbial population involves the use of non-selective media such as D4 medium, Columbia Blood agar (CBA), Trypticase Soy Agar (TSA) which will allow the growth of a wide range of species. As many oral species have complex nutritional requirements supplements such as serum, haemin and menadione are often added to encourage the growth of a wider range of species. Incubation in microaerophilic or anaerobic environments is also necessary for the growth of many oral species. Many of the studies on the composition of plaque associated with gingivitis have used such media (Moore *et al*, 1982; 1984; 1987; Savitt & Socransky, 1984). To enumerate specific species or genera many selective media have been developed for oral species. These include Mitis-Salivarius (MS) agar for the isolation of *Streptococcus* spp., Cadmium Fluoride Acriflavine Tellurite (CFAT) agar for the isolation of *Actinomyces* spp. (Zylber & Jordan, 1982), *F. nucleatum* selective medium (Walker *et al*, 1979), a selective medium for *E. corrodens* (Slee & Tanzer, 1978), *A. actinomycetemcomitans* (Mandell & Socransky, 1981) and oral treponeme isolation medium (OTI), all of which involve the addition of selective agents such as antibiotics and toxins.

Early experimental gingivitis studies relied entirely on cultural techniques. From these studies species such as *A. naeslundii*, *A. odontolyticus*, *F. nucleatum*, *Lactobacillus*, *Streptococcus anginosus* and *Treponema* species were thought to be etiological agents

of gingivitis (Moore *et al*, 1982). These techniques are effective when reliable selective media for a particular species or genera are available and for the initial characterisation of an unknown community. In communities where the genera present are known then selective media can be used to ascertain their numbers. Whilst time consuming, these techniques can also provide quantitative information of the genera within a community giving an idea of the proportion of the community they account for. To detect simple increases and decreases in specific genera where selective media are available these techniques are also useful.

While these techniques have been valuable in isolating many oral species and linking certain species to the aetiology of gingivitis it is clear that with the advance of molecular techniques a vast proportion of oral species cannot be easily cultured, or indeed are currently uncultivable, and thus their contribution to the microbial community as a whole would be significantly underestimated using cultural analysis alone. Identification of bacteria to the species level using culture techniques can be difficult and extremely time consuming for many oral species often involving many biochemical tests or analysis of end products of metabolism using mass spectrometry. Identification of species can be simplified by sequencing of the hypervariable region of the 16S rRNA gene.

1.5.1.2 Culture independent techniques

With the advent of DNA manipulation techniques a whole new discipline emerged: molecular biology. Using a number of techniques based on nucleic acids it is now possible to characterize microbial populations without the need to grow the microorganisms.

1.5.1.2.1 PCR

Conventional PCR has been used extensively to assess the presence of periodontal pathogens in plaque samples. The technique has been particularly applied to species which are difficult to culture or hard to distinguish from other closely related species. For example, specific detection of *A. naeslundii*, *A. viscosus* and *A. odontolyticus* in endodontic samples (Xia & Baumgartner, 2003) has been facilitated using species-specific PCR primers as opposed to culture. This technique has also been applied to detecting periodontal pathogens such as *A. actinomycetemcomitans* and *C. rectus* (Ashimoto *et al*, 1996; van Winklehoff *et al*, 2002) in plaque samples and has revealed that some species are more readily detected using these techniques than by culture alone. Using multiplex PCR more than one organism can be detected at a time. Tran & Rudney (1996) developed a multiplex PCR for the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis* in order to assess the presence of these organisms in supragingival and subgingival plaque samples of healthy individuals. Neither organism was detected in supragingival samples and were very rarely detected in subgingival samples. They further improved on this multiplex PCR (Tran & Rudney, 1999) by including primers for *T. forsythia*, another periodontal pathogen. Using this improved procedure all three organisms were detected in both supragingival and subgingival plaque samples, all organisms being more frequently detected in samples from subjects with periodontitis. Gafan *et al*, (2004) used these same primers to assess the presence of these organisms in gingival crevice plaque from children with and without gingivitis. They detected all three organisms in samples from children with and without gingivitis; interestingly *T. forsythia* was detected more frequently in samples from children without gingivitis. While this method can give us information on whether or not certain bacteria are more commonly detected in plaque samples associated with

health or disease, they do not give information on the numbers of organisms present which may be more indicative of an association between these organisms being actively involved in disease progression.

1.5.1.2.2 DNA-DNA Checkerboard hybridisation

This technique uses species-specific DNA-DNA hybridisation probes to detect large numbers of bacterial species in plaque samples at one time (Socransky *et al*, 1994). Semi Quantitative information on species numbers can be obtained by measuring levels of fluorescence released by probe-DNA interactions. DNA-DNA Checkerboard hybridisation has been used to group oral bacteria into different complexes, each having a different association with health and disease. For example, species from the purple and green complex are thought to be associated with health whilst the orange and red species are linked to the development of gingivitis. The orange complex, comprised of species belonging to the genera *Campylobacter*, *Fusobacterium* and *Prevotella* spp., are most commonly associated with gingival inflammation. The red complex has the strongest association with periodontal disease and includes species such as *P. gingivalis*, *T. forythensis* and *T. denticola.* The technique has been used in several studies to characterise the microbial community of dental plaque. The differences between the microbial composition of supra- and subgingival plaque associated with health and periodontal disease have been assessed using this technique (Ximenez-Fyvie *et al*, 2000b). Interestingly, the key observations made were that *Actinomyces* spp. were the most commonly detected species in all plaque samples regardless of sampling site or disease status. *A. naeslundii* genospecies 2 was far more prevalent than any periodontal pathogens such as *P. gingivalis*, even in sites associated with disease. In contrast, information from culture based studies indicates that *Streptococcus* spp. tend to be the

most abundant species detected, again highlighting that information obtained from culture studies maybe biased to species with the least complex growth requirements (or those that can adapt best to those supplied). The presence of periodontal pathogens such as *P. gingivalis* and *Treponema* spp. as inhabitants of the supragingival plaque associated with health has also been observed (Ximenez-Fyvie *et al*, 2000c; Gmur & Guggenheim, 1994).

The accuracy of bacterial counts obtained using this technique is somewhat unclear as bacterial counts are determined as a comparison to the amount of fluorescence detected from a DNA standard of 10^5 bacterial cells. Furthermore, whatever information is obtained in terms of species proportion is skewed by the original selection of species used as probes. The total amount of DNA needs to be measured with a universal probe in order to gain information on the proportions of the total bacterial community being accounted for using this technique.

1.5.1.2.3 Cloning of the 16S rRNA gene

The small subunit 16S ribosomal RNA gene is present in all bacteria and contains both conserved regions and hypervariable regions. These conserved regions are ideal as targets for universal primers able to detect all bacterial species whilst these hypervariable regions can be sequenced for species identification. The oral cavity supports the growth of a wide range of bacterial species, a significant portion of which are yet to be cultured. The full range of species present has been investigated by amplification of the 16S rRNA present in all bacteria using universal primers, cloning these amplified regions into suitable vectors and sequencing the amplified regions. Paster *et al*, (2001) conducted a study on subgingival plaque samples taken from

subjects with varying degrees of periodontal disease and by sequencing over 2000 clones identified 60% as named species while 40% represented novel phylotypes. Many of these novel phylotypes were found consistently in subjects with disease indicating that using culture techniques alone species which may be significantly linked to disease may not be identified. The figure for the total number of species present in the oral cavity now stands at approximately 630 species (Kazor *et al*, 2003) but this number is likely to still be somewhat conservative.

Munson *et al*, (2004) carried out a similar study on the dental plaque associated with dental caries, but also looked at species which were identified by culture techniques. While fewer species were identified in total from these supragingival plaque samples approximately a third of the species identified had not previously been described. Almost half of the species identified were detected by molecular techniques alone indicating that as well as novel species, those which have more complex growth requirements such *Prevotella* and *Fusobacterium* spp. may not be identified.

There is inherent bias in techniques such as cloning of the 16S rRNA gene, for example, the primers used may not be compatible with all species even though they are thought to be universal. Obviously, this is especially true for species which have yet to be identified as there is no way to test the compatibility of these primers with an unknown species. Furthermore, a common observation with these types of techniques is that some species which are identified by culture are not identified by cloning (Pratten *et al*, 2003a). This may be due to difficulties in extraction of DNA from these bacteria or due to primer bias. For example, bacteria with a high G + C content do not amplify as efficiently with *Taq* polymerase. There is selective amplification of templates with a low G + C content as templates with a high G + C content dissociate into single

stranded molecules with a lower efficiency than those with a low G + C content. This ultimately results in templates with a low G + C content being more efficiently amplified and thus over-represented in the population (Suzuki & Giovannoni, 1996).

The universal primers which are used for 16S rDNA PCR amplification are often degenerate primers (i.e. a mixture of primers differing by one base or more) to allow for variation in the nucleotide sequence of the homologous regions of different bacteria. A single base mismatch results in much lower amplification efficiency in a mixed population. The use of degenerate primers introduces bias as gene fragments with a G or C in the degenerate position will be amplified more efficiently than fragments with an A or T in this position (due to the greater binding energy between G and C than between A and T). This bias is increased under stricter annealing conditions (Kanagawa, 2003).

Regardless of the initial proportions of the 16S rRNA gene of different bacteria in a sample there is a strong bias towards a 1:1 ratio of genes in the final PCR product (Suzuki & Giovannoni, 1996). As PCR products become more abundant rehybridisation can occur interfering with primer binding and extension thus reducing the amplification rate. Therefore, in later PCR cycles less abundant PCR products will be amplified more efficiently. The amount of amplified gene for different species in the final PCR product is not reflective of the number of copies in the starting material. This effect can be reduced by reducing the PCR cycle number (Pratten *et al*, 2003a).

The formation of heteroduplexes (heterologous sequences hybridizing to each other) as the temperature decreases from the denaturing temperature to the annealing temperature in later PCR cycles can cause problems for cloning, sequencing and denaturing gradient

gel electrophoresis analysis due to the formation of artifactual sequences. The mismatch repair action of the *Taq* polymerase enzyme converts the heteroduplex to a homoduplex as DNA is replicated. As the enzyme can use either strand as a template strand for resynthesis of a complementary base the resulting sequence is a mixture of the parent strands. This is an important issue when using cloning of the 16S rRNA gene to examine the richness of species present as some novel sequences could be artifactual sequences and not from novel species. Heteroduplex formation can be avoided by reducing the number of PCR cycles. It is difficult to make any quantitative observations as these biases of PCR cannot be eliminated entirely therefore the main purpose of using this techniques is the potential to identify novel species.

1.5.1.2.4 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was first applied to complex microbial communities by Muyzer *et al*, (1993) for direct determination of their genetic diversity. Briefly, this technique involves amplification of the variable V3 region of 16S rDNA of the entire microbial community by PCR using primers for the conserved regions of the 16S rRNA gene. These amplified fragments can then be separated on the basis of their sequence (Muyzer & Smalla, 1998) as when partially denatured fragments of differing sequences will also have different electrophoretic mobilities through a polyacrylamide gel (Muyzer *et al*, 1993). DGGE has been applied to complex microbial communities such as soil (Gelsomino *et al*, 1999) to get not only a measure of the diversity of species present but also to reveal what the dominant species in these communities might be. As sequence-specific separation of 16S rDNA amplicons of the same length is possible with DGGE a bacterial fingerprint can be generated for a particular ecological sample. As bands can be cut out of the gel and sequenced, novel phylotypes can also be

identified using this technique and this has been applied to the analysis of plaque and saliva communities associated with different forms of periodontal disease (Fujimoto *et al*, 2003; Rasiah *et al*, 2005). Analysis obtained from different individuals has shown that profiles are highly divergent between individuals but remain relatively stable over time for one individual. The intensity of bands however may change over time as disease status changes. Gafan *et al*, (2005) used DGGE to examine the differences in the gingival marginal plaque microbiota of children with and without gingivitis in terms of diversity using the Shannon-Weaver index. They observed greater diversity in samples from children without gingivitis than those with gingivitis, with some bands being found to be significantly associated with each condition by logistic regression analysis. They proposed that this decrease in diversity observed was not necessarily due to there being fewer species present but due to the presence of these species being masked by the increased proliferation of specific species associated with gingivitis. The communities associated with no gingivitis were more stable and therefore supported a more diverse range of species demonstrated by the presence of more bands.

This technique has also been applied to dental plaque microcosm models (McBain *et al*, 2003; Rasiah *et al*, 2005) to assess changes in the microbial communities. Microcosm plaque samples were shown to have more diverse profiles than those obtained from plaque samples (Rasiah *et al*, 2005). As has been observed for plaque samples taken from different individuals, the profiles obtained from replicate *in vitro* communities were also quite different and sequencing of dominant bands showed different species becoming established as the dominant species in different communities.

However, the significance of dominant bands as the most dominant species in a community is not clear due to inherent biases of the PCR technique mentioned earlier. For example, genera such as the *Actinomyces* spp. will always be underestimated in these communities due to having a high G + C content. One of the major drawbacks of this technique is the interpretation of the results, for instance, single bands do not always correspond to a single species. Also, different strains of the same species may not always produce bands with the same migration pattern. This is due to the phenomenon of co-migration (Gafan & Spratt, 2005) of several bands, representing species, to the same position.

1.5.1.2.5 Quantitative PCR

The use of quantitative PCR (qPCR) allows the amount of product in a PCR reaction to be quantified. A PCR reaction can be broken up into three phases, the exponential phase where there is an exact doubling of PCR product at every cycle, the linear phase where reagents start to be consumed and the increase in PCR product is variable with each cycle and the final plateau phase where no more PCR product is being produced. It is during this first exponential phase that the PCR reaction is most specific. In traditional end-point PCR the product is not detected until the final plateau phase and therefore the amount of product may not be proportional to the amount of starting amplification target. Quantitative PCR detects PCR product (in real time) during this exponential phase of DNA amplification when all the reagents are still present (and in excess), the reaction is proceeding at maximum efficiency and the amount of product is directly proportional to the amount of starting material. There are two chemistries utilised for qPCR, SYBR green dye and fluorescent probes. SYBR green is a fluorescent dye which binds to DNA, specifically the minor groove of double-stranded

DNA. Free in solution SYBR green barely fluoresces but when bound to double-stranded DNA fluorescence increases over 100-fold due to conformational changes in the dye (Fig. 1.5.1). The amount of fluorescence emitted is directly proportional to the amount of double-stranded DNA present and thus can be used for quantification by comparisons to the amount of fluorescence emitted from standards of known amounts of DNA. Alternatively fluorescently labelled probes can be used for quantitative detection of PCR product. Using this chemistry an oligonulcleotide probe labelled with a reporter dye and quencher dye is used in conjunction with the PCR primers. In the unbound form the fluorescence of the reporter dye at the 5' end of the probe is suppressed by the close proximity of the quencher dye at the 3' end of the probe. When the probe anneals to the target sequence between the forward and reverse primers it sits in the path of the DNA polymerase enzyme. As the enzyme copies the DNA the 5' exonuclease activity of the enzyme cleaves the probe and the reporter and quencher dyes are no longer in close proximity, so the fluorescence of the reporter dye can be emitted (Fig. 1.5.1). The amount of fluorescence emitted is directly proportional to the amount of DNA that has been copied.

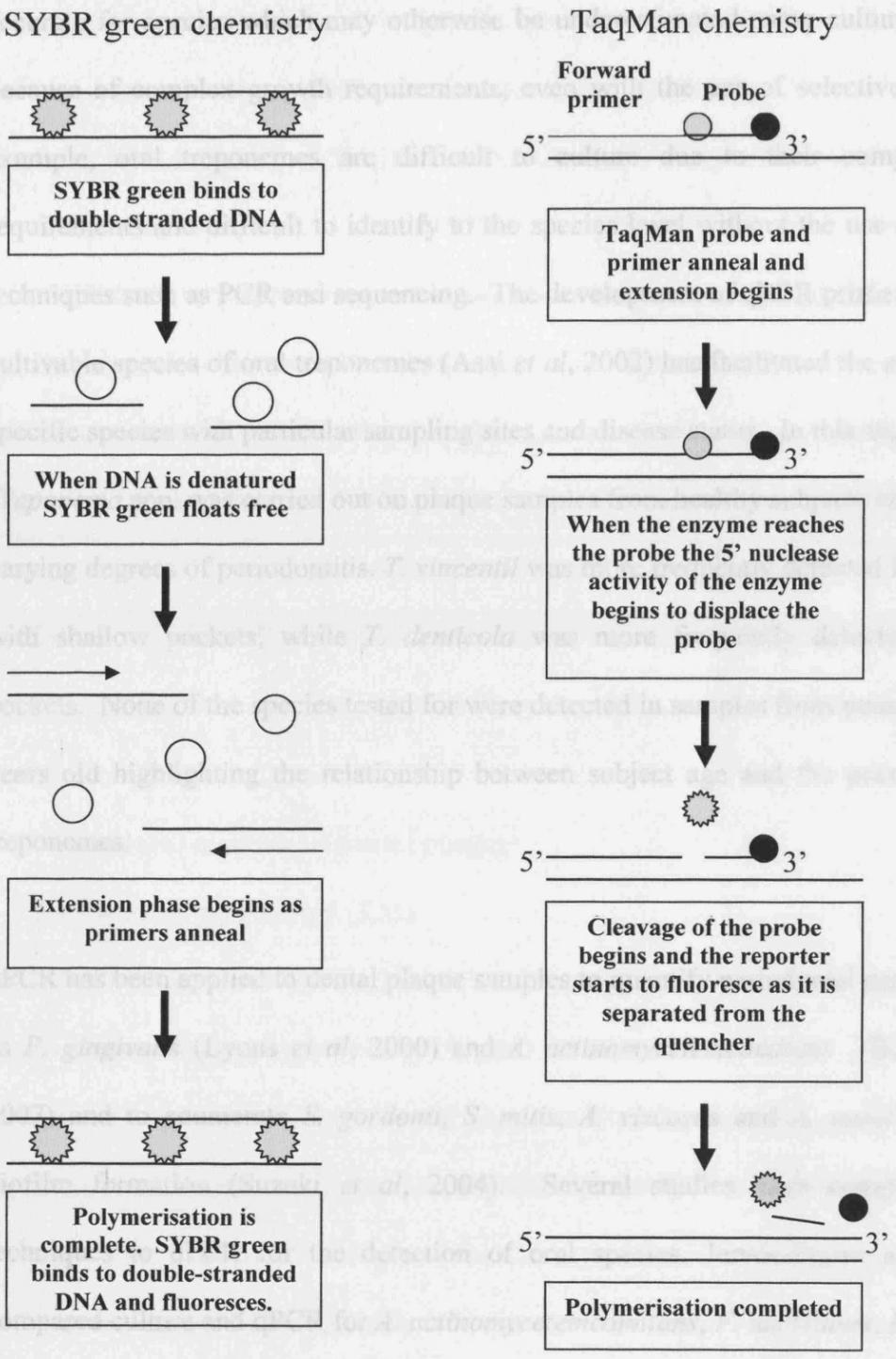

Figure 1.5.1: SYBR green and TaqMan chemistries for quantitative PCR.

The development of qPCR has allowed the rapid assessment of bacterial numbers. By designing species or genera specific primers bacterial numbers can be obtained without the need for time-consuming cultural techniques. Bacterial counts may be more

accurate for species which may otherwise be underestimated using culture techniques because of complex growth requirements, even with the use of selective media. For example, oral treponemes are difficult to culture due to their complex growth requirements and difficult to identify to the species level without the use of molecular techniques such as PCR and sequencing. The development of qPCR primers for several cultivable species of oral treponemes (Asai *et al*, 2002) has facilitated the association of specific species with particular sampling sites and disease status. In this study qPCR for *Treponema* spp. was carried out on plaque samples from healthy subjects and those with varying degrees of periodontitis. *T. vincentii* was more frequently detected from patients with shallow pockets, while *T. denticola* was more frequently detected in deeper pockets. None of the species tested for were detected in samples from patients under 19 years old highlighting the relationship between subject age and the presence of oral treponemes.

qPCR has been applied to dental plaque samples to quantify periodontal pathogens such as *P. gingivalis* (Lyons *et al*, 2000) and *A. actinomycetemcomitans* (Boutaga *et al*, 2007) and to enumerate *S. gordonii*, *S. mitis*, *A. viscosus* and *A. naeslundii* during biofilm formation (Suzuki *et al*, 2004). Several studies have compared culture techniques to qPCR for the detection of oral species. Jervoe-Storm *et al*, (2005) compared culture and qPCR for *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *Pr. intermedia* and *T. forsythia*. They found that culture and qPCR estimates for *A. actinomycetemcomitans* and *P. gingivalis* showed excellent agreement, *T. forsythia* showed fair agreement whilst *Pr. intermedia* and *F. nucleatum* showed poor agreement. *Pr. intermedia* were detected by qPCR in subgingival plaque samples which had tested negative for these organisms by culture (Boutaga *et al*, 2005) and *P. gingivalis* were

detected more frequently and in greater numbers by qPCR in subgingival plaque samples (Boutaga *et al*, 2003). *Actinomyces* spp. (especially *A. naeslundii* and *A. viscosus*) are notoriously difficult to distinguish, even using biochemical tests (Hall *et al*, 1999). However, Suzuki *et al*, (2004) developed qPCR primers to distinguish and quantify these organisms in early biofilm formation using genomic subtractive hybridisation as this technique has been used successfully to identify genomic differences between closely related strains. *A. naeslundii* and *A. viscosus* along with other oral species associated with early biofilm formation were quantified in dental plaque samples from subjects not known to be suffering from periodontal disease. *A. naeslundii* was more frequently detected than *A. viscosus* in the samples tested and in greater numbers.

1.5.2 Structural analysis of dental plaque

1.5.2.1 Electron microscopy (EM)

Both scanning and transmission electron microscopy techniques have been applied to dental plaque biofilms in order to get a greater understanding of their structure. These techniques have been applied to dental plaque to elucidate the process of dental plaque formation from initial colonisation to the development of mature plaque as a physical process. From various studies the structural changes that occur during plaque formation and maturation have been characterised (Listgarten, 1976). During initial attachment the dominant morphologies observed are cocci, most likely streptococci, with few rods present. As plaque matures more rods, filaments and spirochaetes are visualised and a much more heterogeneous structure is observed. Spatial differentiation of species with different atmospheric requirements in oral biofilms has been observed by EM. For example, *N. subflava* was observed in layers closer to the biofilm surface while *F.*

nucleatum, an anaerobic species, was observed in deeper layers (Kinniment *et al*, 1996b). EM has also been used to observe bacterial co-aggregations formed by oral species (Shen *et al*, 2005) and mechanisms of attachment to surfaces and each other. For example, Hongo *et al*, (2007) used EM to observe the fimbriae of *P. gingivalis* cells and their role in the formation of a dense meshwork amongst cells and their possible role in pathogenesis by their ability to adhere to erythrocytes.

Environmental SEM (ESEM) unlike normal electron microscopy allows samples to be examined in a hydrated state and is more rapid and accurate than SEM (Little *et al*, 1991). This technique has been applied to the examination of an ex-vivo biofilm consortium (Vitkov *et al*, 2005b), evaluating the adhesion of oral bacteria on different surfaces (Barbour *et al*, 2007) and the evaluation of endodontic treatments (Bergmans *et al*, 2005).

Atomic Force Microscopy (AFM) measures the attractive forces between a tip and a sample. As well as topographical imaging it is possible to map the surface morphology of biological specimens using this technique (Butt *et al*, 1990). AFM has been used to obtain high resolution topographical images of oral bacteria and to measure the increase in bacterial adhesion forces (Fang *et al*, 2000) and to look at surface structures of *S. mutans* associated with caries (Cross *et al*, 2007). It has also been applied to molecular mapping of bacterial surfaces by the measurement of interactive forces between co-aggregative and non-coaggregative oral bacterial pairs (Postollec *et al*, 2006).

1.5.2.2 Viability staining

Confocal laser scanning microscopy has also been applied to dental plaque to elucidate its structure. Using this technique biofilm structure can be observed in its naturally hydrated state without the need for fixing and staining which is necessary for electron microscopy. From studies using this technique it has been revealed that the plaque architecture is more open than previously thought from observations using traditional electron microscopy techniques and contain water channels linking the deeper layers of the biofilms to nutrients and moisture available at the surface (Wood *et al*, 2000; Auschill *et al*, 2001). The distribution of viable and non-viable bacteria in dental plaque biofilms has also been studied in conjunction with viability stains on both *ex vivo* (Arweiler *et al*, 2004) and *in vitro* biofilms (Pratten *et al*, 2000) to obtain measurements on biofilm thickness (Auschill *et al*, 2001) and to examine the response of biofilms to the application of antibacterial agents (Hope *et al*, 2004).

1.5.2.3 Fluorescent in situ hybridisation

The spatial arrangements of dental plaque bacteria have been studied using fluorescent in situ hybridisation (FISH) in conjunction with confocal laser scanning microscopy in order to get a three-dimensional view of the structures formed by different bacteria. Guggenheim *et al*, (2001b) examined the relationships between five oral species in an *in vitro* biofilm model of dental plaque and observed different interspecies interactions. *F. nucleatum* was observed to interact with all other species in the model, forming structures that were dispersed throughout the biofilm after 40 hours of development. *A. naeslundii* species also formed structures that were more evenly distributed in more mature biofilms. This technique has also been applied to the detection of uncultivable species in dental plaque such as the TM7 group of bacteria (Ouverney *et al*, 2003).

1.6 Modelling dental plaque and plaque-related diseases

1.6.1 *In situ* models

The ideal situation for studying the development of periodontal diseases progression and the response of dental plaque biofilms to potential anti-plaque agents is *in situ*. As such the experimental gingivitis model has been extensively employed where after professional tooth cleaning subjects refrain from dental hygiene for periods of up to four weeks. Several studies have employed the use of *in situ* devices for plaque accumulation which can be removed for plaque analysis. Plaque biofilms have been developed *in situ* using a variety of different surfaces including glass (Auschill *et al*, 2002), human dentine (Wecke *et al*, 2000), bovine enamel (Giersten *et al*, 2000) and human enamel (Macpherson *et al*, 1991; Robinson *et al*, 1997). These types of models are particularly useful for structural studies of plaque as the natural structure can be maintained once removed from the mouth. Wood *et al*, (2000) examined biofilms developed on in situ devices placed on the teeth which were allowed to accumulate biofilms for 4 days. These biofilms were removed and examined using CLSM, allowing the heterogeneous structure to be visualised and the presence of fluid filled voids within the biofilms to be detected. The changes in structure and density of biofilms over time on natural enamel surfaces (i.e. the tooth surface itself) have also been used (Wood *et al*, 2000). *Ex-vivo* samples can be used for testing of antimicrobial agents on naturally developed biofilms in a laboratory setting. For example, gingival epithelial cells with biofilm attached have been used to test the effect of chlorhexidine (Vitkov *et al*, 2005a).

There are several limitations when analysing natural plaque samples due to heterogeneity between individuals, small quantities of sample available, limited access, the variable and uncontrollable nature of the oral cavity and the ethical issues raised

when dealing with human subjects and samples (Anderson *et al*, 2002). To examine what the key environmental factors are in the development of disease there needs to be a means to control these factors which is not possible in an experimental gingivitis model, or samples generated with an *in situ* device. The nutrients available are dependent on the diet of participants and the exact composition and flow rate of an individual's saliva, all factors which cannot be controlled. The way an individual will respond to experimental gingivitis in terms of inflammation and bleeding is another variable which could have a significant effect on microbial composition. Samples taken from a different site in the same individual can differ in composition and thickness as can samples taken from different individuals participating in the same study. The methodologies used for different experimental gingivitis studies such as sampling site and method show great variability making direct comparisons between studies difficult (Spratt & Pratten, 2003).

1.6.2 *In vitro* models

The development of *in vitro* models of the oral environment allows for greater control of influential factors whilst aiming to replicate the natural environment as closely as possible (Sissons *et al*, 1997). In a controlled model environment it is also possible to examine the effect of specific environmental changes on particular species and the community as a whole. Using a controllable system to develop oral biofilms from a known starting inoculum and by using the same inoculum for repeat experiments there is an increased likelihood of getting reproducible results. Laboratory experimental systems developed to study dental plaque range from those involving a single or a few bacterial species to microcosm plaque models.

1.6.2.1 Simple models for dental plaque

The choice of model system used to replicate dental plaque is dependent on its intended use. To study aspects of plaque development simple models can be used to examine the relationships between specific oral species on an orally relevant surface. Studies using simple oral biofilms grown on hydroxyapatite discs in cell culture plates have examined the spatial arrangement of different species and how they interact with each other to form particular structures (Guggenheim *et al*, 2001a) and how macromolecules diffuse through biofilms (Thurnheer *et al*, 2003). Biofilms can be grown on nitrocellulose filters by laying these over an agar base then inoculating with planktonic culture of the desired species. Biofilms developed in this way have been used to evaluate the ability of ozone to kill *E. faecalis* biofilms (Hems *et al*, 2005) and to examine the induction of lethal photosensitization on *Streptococcus pyogenes* biofilms using a confocal scanning laser as the excitation source (Hope & Wilson, 2006).

Microplates have been used to develop caries related microcosms (Filoche *et al*, 2007). They used saliva from two donors as the inoculum and developed biofilms in 24 well microplates using artificial saliva as the growth medium. The addition of sucrose to these biofilms led to increases in species associated with caries. Even with relatively simple models such as these complex community dynamics could be observed. Calcium-hydroxyapatite discs placed in tissue culture plates have been used to develop biofilms from subgingival plaque samples (Walker & Sedlacek, 2007). Using this technique they developed microcosm biofilms which showed similarity to the subgingival plaque samples that they were derived from. This model was used by the same workers (Sedlacek & Walker, 2007) to assess antibiotic resistance in these biofilms. They found that up to a 250 times increase in antimicrobial concentration that

would be required for action against planktonic bacteria was required to have any effect on these biofilms. They also observed that once the biofilms had reached a steady state they became even more resistant.

In vitro models of a few known species have elucidated how specific bacteria form co-adhesive relationships with each other (Ellen *et al*, 1997) highlighting how the increased presence of periodontal pathogens in mature plaque associated with disease is dependent on their ability to attach to species present in the developing plaque. Flow cell experiments have allowed the initial adherence and co-aggregation stages of plaque development to be visualized using models composed of a few key oral species (Foster & Kolenbrander, 2004). The sequential nature of attachment and co-aggregation of species known to form co-aggregative partnerships can be visualised effectively using this method with the use of microscopy and species-specific fluorescent probes as biofilms are generated on saliva coated surfaces. Simple models of dental plaque can be used, however, the biofilms produced using these types of models do not reflect the complexity and diversity of natural plaque (Filoche *et al*, 2007).

1.6.2.2 Complex models of dental plaque

In more complex models of dental plaque the manipulation of environmental factors can occur. As these factors can be controlled they can mimic the natural system as closely as possible. Dental plaque microcosms are oral biofilms developed *in vitro* from material sampled from the oral cavity such as plaque or saliva samples (Wimpenny, 1997). As these communities are derived from the natural microbiota the complexity and heterogeneity observed in natural plaque is better replicated in these models as the communities are allowed to evolve from the original starting material present in the

mouth (Sissons, 1997). The obvious advantage to using these samples as opposed to natural plaque samples is convenience and the precise control and measurement of environment parameters that is not possible in the natural environment. The concept of using dental plaque microcosms is not a new one, an early example being the use of sections of tooth immersed in saliva for the growth of microcosm plaque (Dietz, 1943). Systems which have been developed for the study of biofilms developing in different environments have been applied to the study of oral biofilms.

1.6.2.2.1 The Robbins device

The Robbins device was developed by McCoy *et al*, (1981) and consists of removable plugs inserted in a tube through which liquid inoculum and growth medium flow. Biofilms form on these removable plugs and are subjected to continuous shear forces as growth medium flows through. As this flow rate can be controlled the amount of shear these biofilms are exposed to can also be controlled. The growth rate of biofilms can also be controlled and measured. This method has not often been applied to the development of oral biofilms, its use mainly being restricted to testing the antimicrobial susceptibility of biofilms of a single oral species such as *S. sanguinis* (Larsen & Fiehn, 1995) and *P. gingivalis* (Larsen, 2002). It has also been applied to testing novel anti-plaque agents against *S. mutans* biofilm formation (Honraet & Nelis, 2006).

1.6.2.2.2 The chemostat

The chemostat model allows factors such as growth medium, inoculum, pH and atmosphere to be altered. Most importantly the growth rate of biofilms can be controlled and measured. By adjusting the dilution rate in a chemostat, reproducible steady-state conditions at a certain exponential growth rate are obtained because the

physiological state of bacteria, i.e., the number of cells, growth rate, and surrounding environment, is stable. The growth rate of bacteria and the dilution rate become equal in the steady state. With these characteristics, this method is ideal for studying the physiology of bacteria (Masuda *et al*, 2006). Bradshaw *et al*, (1996a) used a chemostat fed by another chemostat under different atmospheric conditions to develop a model community of ten oral species. The bacterial succession observed during *in vivo* plaque formation was mimicked using this model as communities were initially dominated by aerobic species such as *Neisseria* spp. with obligate anaerobes only became established in more mature plaque after 4-7 days. This model has been used to examine biofilm formation in microcosms (Sissons, 1997) and known species consortia (Bradshaw *et al*, 1996b) and to assess the influence of anti-plaque agents on dental plaque (Herles *et al*, 1994). It has also been used to demonstrate how interactions between oral bacteria allow the survival of strict anaerobes in an aerated environment (Bradshaw *et al*, 1998). The major disadvantage with this technique is that biofilm thickness is not maintained and a high proportion of the community will be planktonic cells and not biofilm cells due to the fluid phase of the chemostat.

1.6.2.2.3 The artificial mouth

The artificial mouth model has been used to develop microcosm biofilms using saliva as the inoculum to assess artificial saliva formulations (Wong & Sissons, 2001) for large-scale biofilm culturing, the influence of urea and sucrose on plaque pH (Sissons *et al*, 1992; 1994) and to assess the influence of different chemical agents on microbial viability (Sissons *et al*, 1996). The model allows the control and measurement of substratum, nutrient source, pH and redox potential but as with chemostats the major drawback is the lack of control of biofilm thickness. A limited number of samples can

be produced and biofilm growth is unrestrained so the biofilms that form independently in separate chambers are not highly reproducible.

1.6.2.2.4 The multiple Sorbarod device

More recently, the multiple Sorbarod device (MSD) has been used to develop perfused oral microcosms on filters (McBain *et al*, 2005) using freshly collected saliva as the inoculum. The biofilms that develop have been shown to be representative of dental plaque communities by checkerboard DNA-DNA hybridisation techniques. The composition of the communities that developed was shown to be highly dependent on the composition of the freshly collected saliva used as the inoculum. However, this technique is not suitable for testing of dental materials or substrata.

1.6.2.2.5 The Constant Depth Film Fermenter

The Constant Depth Film Fermenter (CDFF) was developed by Peters & Wimpenny (1988) initially to test the effect of biocides on biofilms (Kinniment & Wimpenny, 1992) and to assess *Pseudomonas* biofilms (Wimpenny *et al*, 1993) before becoming established as a representative model of the oral cavity (Marsh *et al*, 1995; Wilson *et al*, 1995; Pratten *et al*, 2003a), allowing the control of key parameters including nutrient source, temperature, pH, oxygen availability and substrata. The CDFF model displays reproducibility between samples and it allows the limitation of biofilm thickness by mechanical shear forces similar to the natural shear forces that would limit plaque accumulation in the mouth. The depth of biofilms generated can be altered by recessing discs of the desired substratum to a specified depth (see Chapter 2 for details on setup). A large number of replicate samples (up to 75) are produced using this model which can be removed aseptically at any time point during an experiment for various analyses. The

CDFF can also be kept running for potentially unlimited periods of time. This feature is particularly advantageous when testing longer term effects of anti-plaque agents on plaque composition and control. Initial studies involved feeding the CDFF with a bacterial consortium of key oral species from a chemostat (Marsh *et al*, 1995). All species could survive using this model; initially aerobic species such as *Neiserria subflava* were dominant, followed by increased levels of anaerobic species as the plaque matured, as would be seen with naturally developed plaque *in vivo*. The CDFF is well established as an *in vitro* model for supragingival plaque and as is demonstrated in Table 1.4.1 has been applied to the evaluation of oral biofilms for a variety of purposes.

When modelling periodontal diseases this model allows oral biofilms to become established under a defined set of conditions then be exposed to new environmental challenges to examine changes that occur in these steady-state communities. For example, exposure to anti-plaque agents has been shown to alter the microbial composition of stable oral communities (Pratten *et al*, 1999; Ready *et al*, 2002). If the same inoculum is used there is a good level of reproducibility between repeat experiments and thus meaningful comparisons between experiments where specific environmental conditions have been altered.

Application	References
Model of dental plaque biofilms	Marsh *et al*, 1995b; Wilson, 1995
Assessment of agents for the control of oral biofilms	
Chlorhexidine	Kinniment *et al*, 1996; Pratten *et al*, 1998a;b; Pratten *et al*, 1999; Mc Bain *et al*, 2003b; Deng *et al*, 2004a; Hope *et al*, 2004; Leung *et al*, 2005
Tetracycline	Ready *et al*, 2002; Wirthlin *et al*, 2005
Anti-fungals	Lamfon *et al*, 2004; Lamfon *et al*, 2005
Fluoride	Embleton *et al*, 1998; Bradshaw *et al*, 2002; Badawi *et al*, 2003
Silver ions	Mulligan *et al*, 2003
Mechanical cleaning	Hope & Wilson, 2002b; Hope *et al*, 2003; Hope & Wilson, 2003b
Photodynamic therapy	Wilson *et al*, 1996; O'Neill *et al*, 2002; Zanin *et al*, 2005; Metcalf *et al*, 2006; Wood *et al*, 2006
Transfer of genetic elements in oral biofilms	Roberts *et al*, 1999; Roberts *et al*, 2001
Structural studies of oral biofilms	Vroom *et al*, 1999; Pratten *et al*, 2000; Hope *et al*, 2002; Hope & Wilson 2003a
Characterising species composition and diversity of oral biofilms	Pratten *et al*, 2003a; McBain *et al*, 2003a
Modelling plaque-related diseases	
Endodontic microleakage	Martharu *et al*, 2001
Oral malodour	Pratten *et al*, 2003b
Caries (De- and remineralization of dentin)	Deng *et al*, 2004b; Deng *et al*, 2005
Denture associated stomatitis	Lamfon *et al*, 2005

Table 1.6.1: The various applications of the CDFF in studying oral biofilms

1.7 Aims of research

Many models ranging in complexity have been developed for supragingival plaque but a specific *in vitro* model for gingivitis associated plaque has not been developed as yet.

1) The first aim of this research is to develop a reproducible model to study the growth of supragingival plaque specifically associated with gingivitis using the constant depth film fermenter.

2) The second aim is to characterize the microbial communities that develop in this model using a variety of techniques including traditional culture and molecular methods.

3) The final aim is to develop antimicrobial and anti-plaque coatings on relevant dental materials to assess the influence these agents have on the microbial communities present in this model.

CHAPTER 2

Materials and Methods

2.1 The Constant Depth Film Fermenter

The Constant Depth Film Fermenter (CDFF) was originally developed by Peters and Wimpenny (1988). Those used for the course of this work were produced by John Parry-Jones Engineering (Cardiff, UK) and the operating conditions used for the development of single-species and microcosm dental plaque biofilms were modifications of methods described previously (Wilson *et al*, 1995; Pratten *et al*, 1998b; Pratten *et al*, 1999; Pratten and Wilson, 1999; Pratten *et al*, 2003).

2.1.1 CDFF Setup

The CDFF (Fig. 2.1.1) consists of a stainless steel turntable contained within a glass vessel by a stainless steel top plate and base plate, retained by polytetrafluoroethylene (PTFE) seals and high vacuum silicone grease (Dow Corning, Wiesbaden, USA). The top plate contains inlets for growth media and atmospheric gases and a sampling port for the removal of biofilms during an experimental run. The base plate contains an outlet for effluent. The turntable itself holds up to 15 PTFE sampling pans which are positioned flush with the rim of the turntable. Each sampling pan has five cylindrical holes (5 mm diameter) which hold PTFE plugs upon which discs of appropriate substrata can be held and recessed to a desired depth. The turntable is attached to a detachable motor (MK III Fermenter, University College Cardiff Consultants Ltd., UK) via a central spindle.

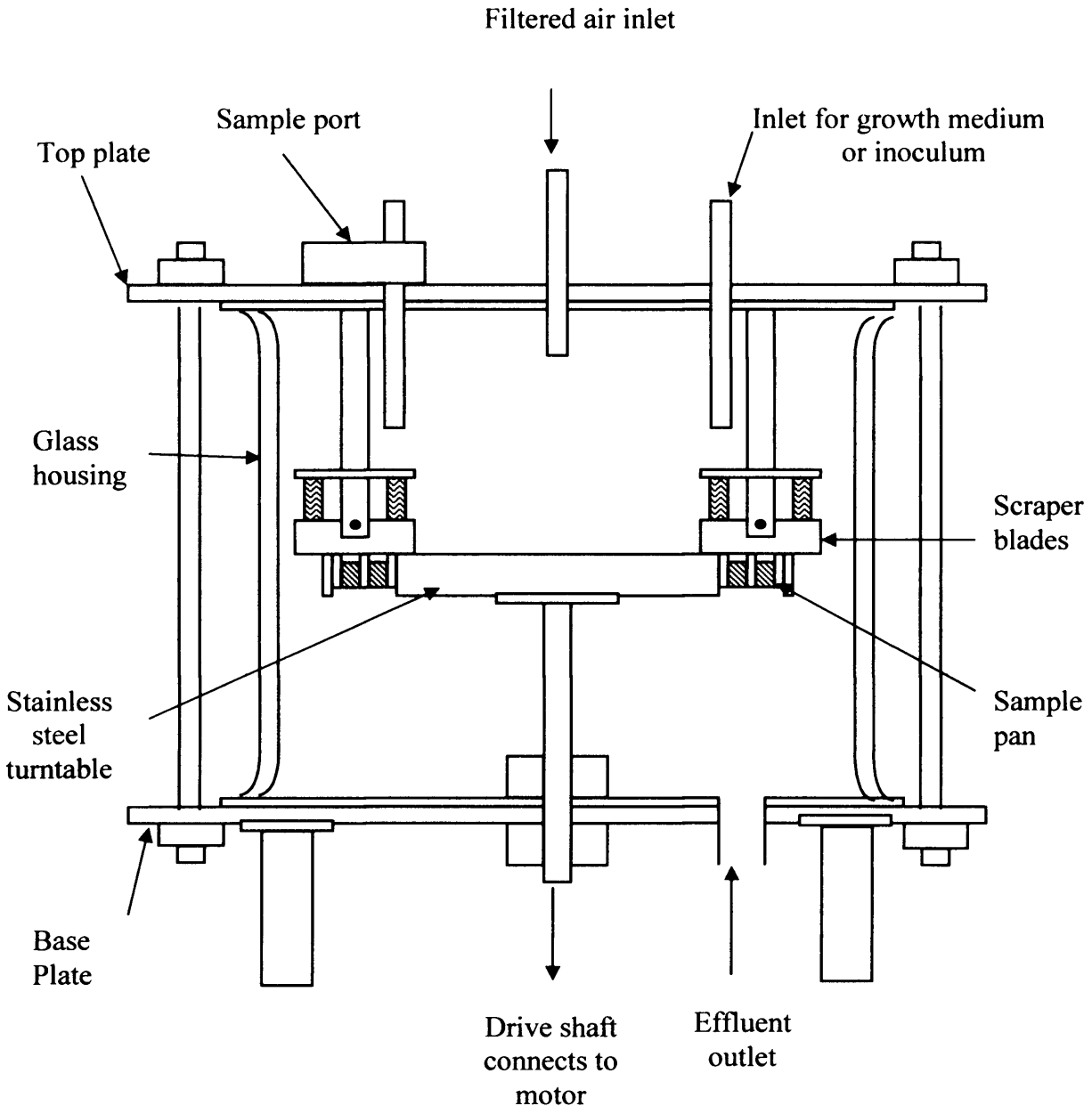

Figure 2.1.1: The Constant Depth Film Fermenter (CDFF):

Schematic vertical section through the fermenter

The turntable rotates at a constant rate as growth media are pumped in via the inlets in the top plate. Attached to the top plate are two scraper blades which sweep the inocula and growth media into the recesses and once biofilms are formed prevents them from exceeding the desired depth (Fig. 2.1.2).

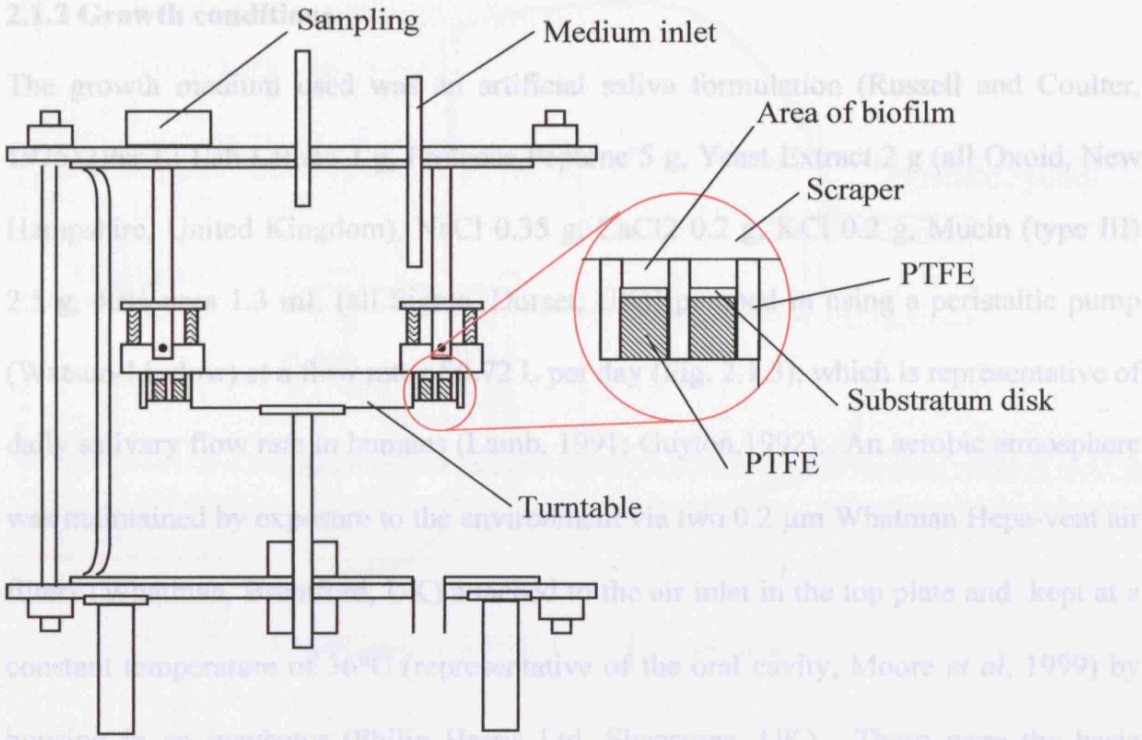

Figure 2.1.2: Growth of biofilms in the CDFF.

Silicone tubing (Fisher Scientific Ltd., UK) was attached to the inlets in the top plate and the outlet for effluent in the base plate and secured in place using cable ties. This tubing was fitted with connectors which could be attached to tubing coming from growth medium reservoirs or the inoculum and also for connection to the waste medium vessel. Marprene tubing (Fisher) was used to for sections of tubing required to run through a peristaltic pump (Watson-Marlow, Falmouth, UK) in order to maintain the flow rate at the desired setting.

Once assembled the entire CDFF and attached tubing could be sterilised in an autoclave at 121°C (pressure 15 psi) for 15 mins.

2.1.2 Growth conditions

The growth medium used was an artificial saliva formulation (Russell and Coulter, 1975) [Per L: Lab Lemco 1 g, Proteose Peptone 5 g, Yeast Extract 2 g (all Oxoid, New Hampshire, United Kingdom), NaCl 0.35 g, CaCl2 0.2 g, KCl 0.2 g, Mucin (type III) 2.5 g, 40% urea 1.3 mL (all Sigma, Dorset, UK)] pumped in using a peristaltic pump (Watson-Marlow) at a flow rate of 0.72 L per day (Fig. 2.1.3), which is representative of daily salivary flow rate in humans (Lamb, 1991; Guyton,1992). An aerobic atmosphere was maintained by exposure to the environment via two 0.2 μm Whatman Hepa-vent air filters (Whatman, Brentford, UK) attached to the air inlet in the top plate and kept at a constant temperature of 36°C (representative of the oral cavity, Moore *et al*, 1999) by housing in an incubator (Philip Harris Ltd. Shenstone, UK). These were the basic conditions for biofilms growing under conditions emulating health.

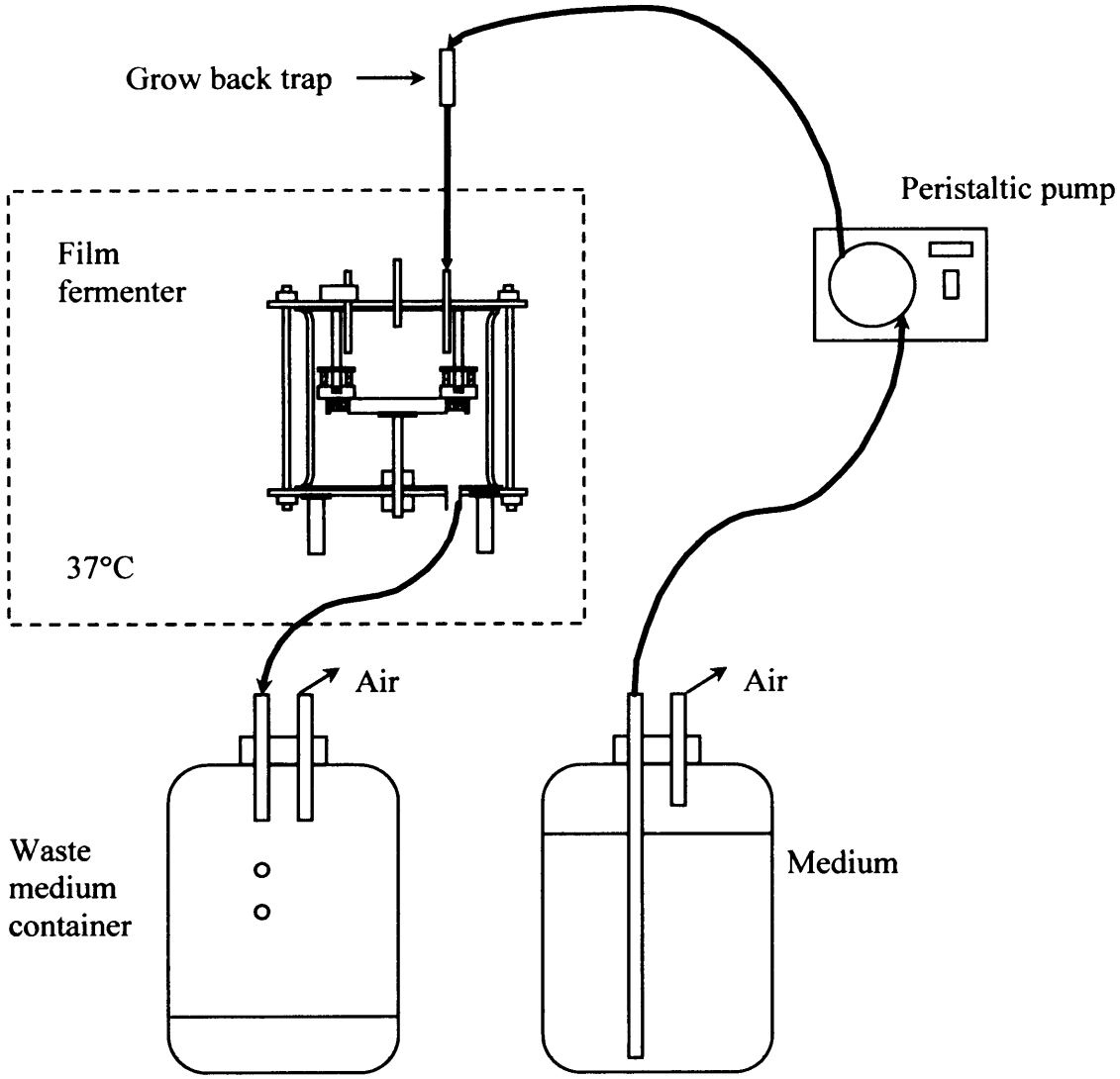

Figure 2.1.3: CDFF setup.

2.1.3 Inoculation of dual-species biofilms

A. naeslundii NCTC 10301 and *Streptococcus sobrinus* NCTC 12279 were grown anaerobically in brain heart infusion broth (BHI; Oxoid) at 37°C to a concentration of approximately 1 x 10^8 CFU ml^{-1}. 10 ml of this culture was added to 500 ml of artificial saliva then pumped into the CDFF at a continuous rate of 1 ml min^{-1} for 8 hours using a peristaltic pump (Fig. 2.1.4).

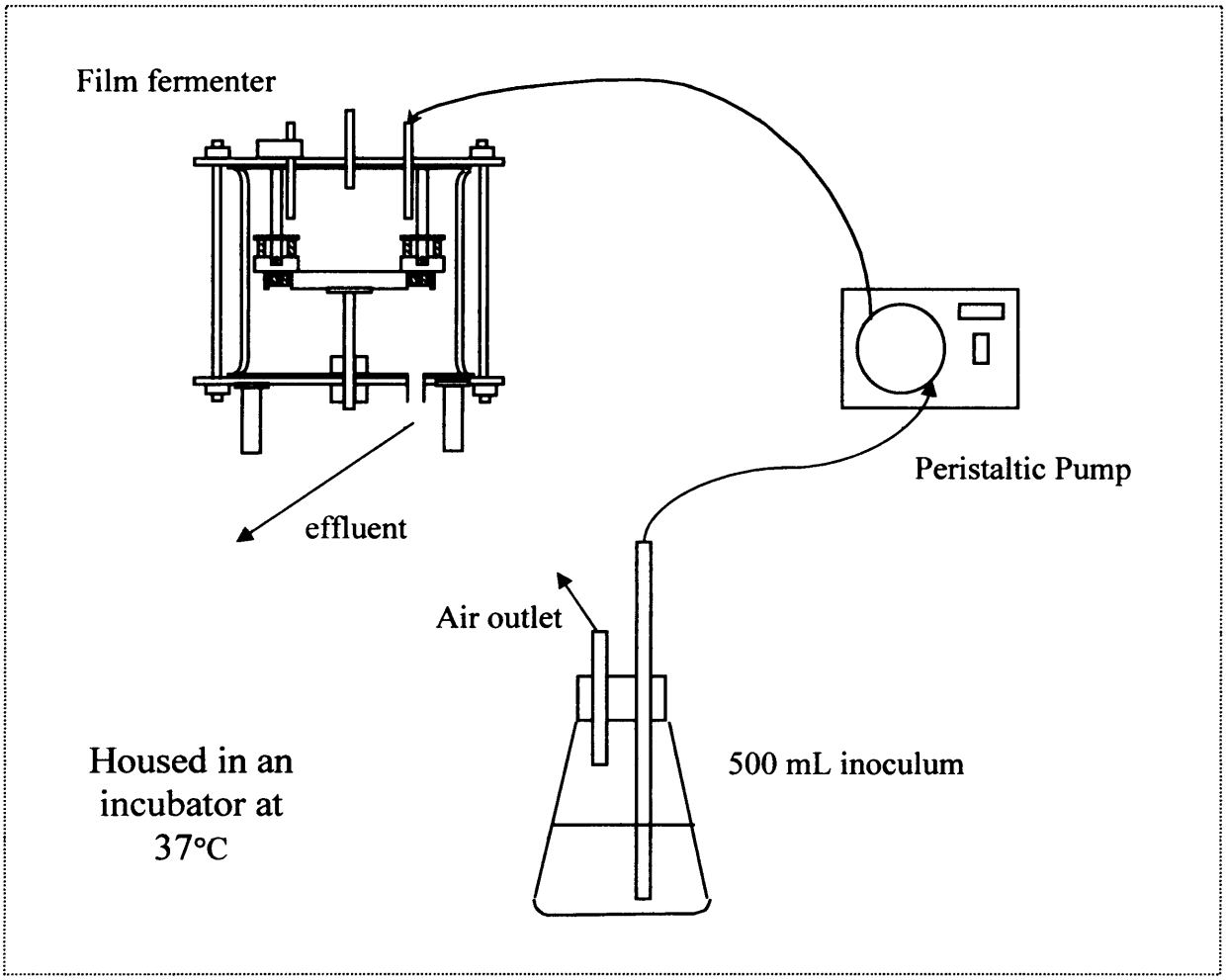

Figure 2.1.4: Inoculation of the CDFF.

2.1.4 Inoculation of microcosm biofilms

In order to produce microcosm biofilms, whole saliva was obtained from fifteen (8 female, 7 male) healthy individuals and used to create a pooled stock to be used as the inoculum for each experiment. The volunteers were all between the ages of 20 and 50 and were not under any form of antibiotic treatment at the time of sampling. All of the volunteers were non-smokers and samples were taken prior to eating. Equal amounts of saliva from each individual were added to the pool along with glycerol (final concentration, 10% v/v). This pool was then split into 1 ml aliquots which were stored at -80°C. These 1 ml aliquots were added to 500 ml of artificial saliva then pumped in to the CDFF at a constant rate of 1 ml min^{-1} under microaerophilic conditions (2% O_2, 3% CO_2, 95% N at 200 bar; BOC Gases, Guildford, UK) via a filtered air inlet in the top plate at a rate of 200 cm^3 min^{-1} for the 8 hour inoculation period before switching back to an aerobic atmosphere once inoculation was ceased.

2.1.5 *In vitro* model parameters

After initial comparisons of bovine enamel and hydroxyapatite as substrata for bacterial colonisation there was no difference in the total counts or species proportions and as such hydroxyapatite discs (5 mm diameter) were used throughout the rest of the study. Hydroxyapatite is an appropriate substitute for dental enamel (Wheeler *et al*, 1979; Ciardi *et al*, 1987) and is easier to obtain than bovine enamel. The discs were recessed to a depth of 600 μm using a specially engineered tool. This recess depth was used to allow biofilms to develop which were representative of plaque observed in the interdental regions of supragingival plaque associated with poor dental hygiene (Newman & Morgan, 1980). Biofilms were removed aseptically from the CDFF during the course of each run via the sampling port and subjected to various analyses.

2.1.6 Gingivitis conditions

To provide nutrients associated with the onset of gingivitis an artificial GCF formulation (Wilson, 1999) comprised of 60% RPMI tissue culture medium (which was used here to provide nutrients present in tissue exudates which are a major component of GCF), 40% horse serum, 0.5 μg ml^{-1} menadione and 5.0 μg ml^{-1} haemin (all Sigma) was used. This was pumped into the artificial saliva formulation as it flowed into the CDFF at a rate of 50 μl min^{-1}, as an approximation of the flow rate of GCF into saliva during gingivitis (Goodson, 2003). This was accompanied by a switch to a more microaerophilic environment by pumping in a microaerophilic gas mixture (2% O_2, 3% CO_2 , 95% N at 200 bar) with an oxygen content associated with periodontal disease (Loesche *et al*, 1983) via a filtered air inlet in the top plate at a rate of 200 cm^3 min^{-1}.

2.1.7 Sampling of the CDFF

At various time points PTFE sample pans each containing 5 biofilms were removed aseptically from the CDFF. This was done by lining up the pan to be removed with the sampling port and stopping the rotation of the turntable by switching off the motor. The sample port was then flamed with a portable bunsen burner and sprayed with 70% ethanol in order to keep the area sterile. The sample port was then opened and a pre-sterilised sampling tool was used to remove a sample pan by screwing into the central threaded hole of the sample pan. The removed sample pan was subsequently placed in a sterile universal tube (Sarstedt) and individual discs removed using pre-sterilised forceps and placed into an appropriate vessel depending on the type of analysis to be performed.

2.2 Cultural analysis

After aseptic removal from the CDFF biofilms were placed in 1 ml of sterile phosphate buffered saline (Oxoid) containing 5 glass beads (212-300 μm diameter, Sigma) and vortexed for 1 min to create a homogeneous suspension. This suspension was then serial diluted and plated onto appropriate media to give viable counts and species proportions. Serial dilutions were plated onto Fastidious anaerobe agar (FAA; Bioconnections, Leeds, UK) to give total anaerobic counts and on Columbia blood agar (CBA; Oxoid) to give total aerobic counts. *Actinomyces* spp. were selected for on Cadmium Fluoride Acriflavine Tellurite (CFAT) plates (Zylber and Jordan, 1982) and *Streptococcus* spp. were selected for on Mitis-Salivarius agar (MS; BD Biosciences, Oxford, UK). For microcosm plaques, gram-negative species were selected for on CBA with a gram-negative supplement (GN; Oxoid), *Veillonella* spp. were selected for on Veillonella agar (VA; BD Biosciences) and *Lactobacillus* spp. on Rogosa agar (RA; Oxoid). All plates were incubated anaerobically at 37°C for four days except FAA, CFAT and gram-negative selective agar which were incubated for up to two weeks to allow slower growing colonies to become distinct. CBA plates were incubated aerobically at 37°C overnight. On all selective media species were confirmed by colony morphology and gram reaction.

2.3 Identification of cultivable species by 16S rRNA PCR

2.3.1 Selection of isolates for identification

From selected time points during each CDFF experiment isolates were selected for 16S rRNA sequence identification. Serial dilutions on the various selective and non-selective media of the pooled saliva inoculum, samples of the inoculum taken after the inoculation period and of samples removed from the CDFF during the course of an experiment were used for the selection of isolates to be identified. Plates were selected with an appropriate number of colony forming units (between 30 and 300). From the selective media (MS, CFAT, RA, VA and GN) the different colony morphotypes were characterized and enumerated. Distinct colony morphotypes were subcultured onto the respective media to ensure purity. From non-selective media (FAA, CBA) 15 random isolates were subcultured for identification. The different colony morphotypes were also noted and enumerated.

2.3.2 16S rRNA PCR

Oligonucleotides were synthesized by Sigma-Genosys (Pampisford, UK). Well separated colonies were picked with a sterile toothpick and suspended in 50 µl of molecular grade H_2O (Sigma). To this 50 µl of PCR master mix was added to give a total reaction volume of 100 µl. The PCR master mix consisted of 26.7 µl of molecular grade H_2O (Sigma), 10 µl of 10X buffer (Bioline, London, UK), 5 µl of $MgCl_2$ (Bioline), 2 µl of 10 µM dNTP mixture (Bioline), 3 µl each of primers 27F and 1492R (10 µM, Sigma-Genosys) and 0.3 µl of BioTaq (BIOLINE). This was then run on the following PCR program, an initial step of 94°C for 5min, followed by 29 cycles of 94°C for 60 s, 54°C 60 s and 72°C for 90 s and a final extension step of 72°C for 5 min (Biometra T3000, Goettingen, Germany).

2.3.3 Agarose Gel Electrophoresis

PCR products were confirmed by agarose gel electrophoresis. Gels were made up with 1 x TAE (50X TAE Fisher, UK, diluted in distilled H_2O) according to the volume required. A standard concentration of 1% agarose (Amresco, Ohio, USA) was used. The mixture was heated in a glass conical flask in a microwave (Sharp Compact) until the agarose had completely dissolved. The mixture was cooled but still molten before adding 0.5 μg/ml of Ethidium Bromide solution (Sigma). The agarose was poured into a gel tray and allowed to cool to form a gel.

DNA samples were mixed with 5 x Blue Loading Buffer (Bioline) and loaded into the gel wells along with a lane containing Hyperladder II (Bioline) for fragments between 50bp and 2kb. The gels were run at 100V for an appropriate length of time depending on the size of the gel. DNA was visualized in the gels by exposure to UV light in an Alpha Imager (Alpha Innotech Corporation, California, USA) and a photographic record was made of the results (Alpha Imager 1220 Documentation and Analysis System).

2.3.4 Purification of PCR samples

PCR products were purified from the other components in the reaction such as excess primers, nucleotides, DNA polymerase, and salts, using the QIAquick PCR purification kit (Qiagen, Crawley, UK). Each sample (100 μl) was applied to the centre of a GenElute PCR Clean-up miniprep column (Qiagen) followed by 500 μl (5 volumes) of PB buffer (Qiagen), then centrifuged (Eppendorf, Hamburg, Germany) for 1 min at 14,000 rpm (15,800 x g). The flow-through was discarded and 750 μl of diluted PE buffer (55 ml of Qiagen PE buffer diluted with 220 ml of C_2H_5OH 100%) was added, the sample was centrifuged for 1 min at 14,000 rpm (15,800 x g). The flow-through was

discarded and the sample was centrifuged again for 1 min. The column was transferred to a fresh eppendorf tube, 30 µl of Elution Buffer (Qiagen) was applied to the centre of the column and each tube was kept at room temperature for 1 min. The column was then centrifuged for 1 min to give 30 µl of eluted DNA. The sample was run on a 1% agarose gel to confirm the presence of DNA before sequencing.

2.3.5 DNA Sequencing

The sequencing of PCR products was carried out according to the PE Biosystems (Warrington, UK) protocol with the following modifications. The PCR reaction consisted of 1 µl (5 pmol) of primer 357F mixed with 2 µl of a 1/4 dilution of ABI BigDye Terminator Ready Reaction Mix (Applied Biosystems, Warrington, UK) diluted with 5 x sequence buffer (400 mM Tris-HCL and 10 mM $MgCl_2$), 1 to 4 µl of DNA sample and an appropriate volume of molecular grade H_2O (Sigma) to make up the total volume to 7 µl. The samples were then run on the following program (Biometra T3000); 99 cycles of 95°C for 10 s, 50°C for 5 s and 60°C for 4 min followed by a final step of 4°C until samples were removed from the PCR machine.

2.3.6 Ethanol Precipitation of Sequence PCR Products

13 µl of H_2O, 2 µl of 3M sodium acetate (BDH) and 50 µl -20°C 95% ethanol (100% AnalaR BDH, diluted with sterile H_2O) was added to each PCR tube. They were incubated on ice for 20 min. The samples were then transferred to a 1.5 ml eppendorf and spun at 14,000 rpm (15,700 x g, Eppendorf 5402) for 25 min at 4°C. Following that, the supernatant was drained onto 3 mm Whatman filter paper (Whatman, Brentford, UK), and the pellet washed with 250 µl -20°C 70% ethanol then re-precipitated by centrifugation for 15 min at 14,000 rpm (15,700 x g). The supernatant was drained as before and then the tubes were left with their lids open at room temperature to allow the

ethanol to evaporate. Samples were then resuspended in 15 µl of template suppresser reaction buffer (Applied Biosystems), vortexed for 20 s then heated at 95°C for 5 mins. Samples were then transferred to 0.5 ml genetic analyser tubes (Applied Biosystems) and then analysed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Loading of the genetic analyzer was carried out by Dr. Adam Roberts and Miss Tracey Moss (Division of Microbial Diseases, UCL Eastman Dental Institute).

2.3.7 Analysis of sequences

The sequences generated (up to 500 bases) were checked using the CHROMAS program (v.1.43) then compared with the database at BLAST at the National Centre for BiotechnologicalInformation (Altscul *et al.*, 1997, www.ncbi.nlm.nih.gov/BLAST/) for sequence matches in order to find the closest match with the 16s rRNA gene.

2.4 Extraction of Genomic DNA from saliva and plaque samples

DNA was extracted from pooled saliva and plaque samples collected from the CDFF following the protocol for 'Gram-positive bacterial culture' using the Puregene Kit DNA extraction kit for Yeast and Bacteria (Gentra, Nottingham, UK). Cells were pelleted from 1 ml saliva by centrifugation at 13,000 rpm (15,700 x g, Eppendorf 5415D) in 1.5 ml sterile eppendorfs (Sarstedt). Pellets were gently resuspended in 600 µl of Cell Suspension Solution provided with the kit. 3 µl of Lytic Enzyme Solution (4,000 U/ml) was added. The samples were then inverted 25 times and incubated at 37°C for 30 min to digest cell walls. After centrifugation of the samples for 5 min at 13,000 rpm (15,700 x g) in a bench-top centrifuge the supernatant was removed and the cells were suspended in 600 µl of Cell Lysis Solution (Puregene) and gently pipetted up and down to digest the cell walls. The samples were heated at 80°C for 5 min for complete cell lysis. After allowing the samples to cool to room temperature 3 µl of RNAse A Solution (4 mg/ml) (Puregene) was added to the cell lysate. The samples were then mixed by inverting the tubes 25 times and then incubated at 37°C for 1 h to remove the RNA. After cooling the samples to room temperature, 200 µl of Protein Precipitation Solution (Puregene) was added to the cell lysate then vortexed vigorously at high speed for 20 s to ensure uniform mixing. The samples were then left on ice for 15 mins to allow the mixture to become cloudy. The samples were centrifuged for 3 minutes at 14,000 rpm (15,700 x g) allowing the precipitated proteins to form a tight white pellet and the supernatant containing the DNA was poured into a clean 1.5 ml sterile eppendorf containing 600 µl 100% isopropanol (Sigma). The samples were inverted gently 50 times and centrifuged for 5 min at 14,000 rpm (15,700 x g). The supernatant was poured off onto 3 mm Whatman paper (Whatman, Brentford, UK). The pellet was then washed by adding 600 µl 70% ethanol and centrifuged for 5 min at

14,000 rpm (15,700 x g). The tubes were drained on 3 mm Whatman paper and allowed to air dry for 30 min. The DNA pellet was rehydrated in 50 µl DNA hydration solution (Puregene) and incubated for one hour at 65°C then left overnight at room temperature. Samples were then stored at -20°C until used.

2.5 Quantitative PCR analysis of biofilm samples

2.5.1 Preparation of DNA

As the amount of DNA available for analysis was limited (only 50 µl per sample), so DNA samples were diluted by a factor of ten in DNA hydration buffer (Gentra) to provide more sample to work with.

2.5.2 Preparation of DNA to use for standard curve generation

Depending on the specific target of the primers used for each PCR assay a standard curve of bacterial genomic DNA from a known number of bacterial cells was created. Cultures of the specific organism to be used were grown up in BHI broth either aerobically or anaerobically depending on the species. The bacterial numbers in these cultures was then enumerated by plating out of serial dilutions in PBS on either CBA or FAA media. The DNA was then extracted from 0.5 ml of this culture using the Gentra protocol to give extracted DNA corresponding with a known number of bacterial cells. This DNA was then diluted appropriately to give a concentration of 1×10^7 bacteria/ml. This was then serially diluted in DNA hydration buffer to give tenfold decreasing concentrations of DNA corresponding to bacterial numbers.

2.5.3 qPCR plate setup

Quantitative PCR reactions were carried out in 96 well Optical Reaction plates (ABI). It was essential to have enough points to generate a standard curve (at least six dilutions of DNA) and each sample needed to have at least three replicate wells to ensure the accuracy of pipetting. It was also essential to have control wells which contained no template to ensure the purity of all the reagents used. Therefore on each plate, taking into account the space taken up by standard curves, template controls and the need for at

least three replicates of each sample, there was enough space to process a maximum of 24 separate samples of extracted DNA.

In each well there was 25 µl of reaction mix. This consisted of 8.0 µl of POWER SYBR Green (ABI) PCR master mix, 0.25 µl of each primer (varying concentrations), 2.5 ml of DNA and 14.0 µl of molecular grade H_2O (Sigma) to make up the volume to 25 µl. To ensure maximum reproducibility between replicates a separate master mix was made for each sample to be tested.

Once the plate was setup the plates were sealed closed using plastic plate seals (ABI). The plates were then spun for 5 s in a plate centrifuge (Eppendorf 5810R) to ensure that there were no bubbles at the bottom of any of the wells.

2.5.4 Loading the PCR plate and PCR cycle

The running of each qPCR reaction needed to be setup with the 7300 System Software for the qPCR machine (ABI Prism 7300). Using the software the contents of each well could be labelled and replicate samples grouped together. For the wells containing DNA for the production of standard curves, the corresponding number of bacteria needed to be entered into the plate setup program.

The standard PCR conditions used for qPCR were an initial step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s, followed by a final dissociation stage of 95°C for 15 s, followed by 60°C for 30s and 95°C for a further 15s. The data collection step was the 60°C step in each round of 40 cycles.

2.5.5 Analysis of qPCR results

2.5.5.1 Amplification plots

The amount of fluorescence detected by the qPCR machine as the reaction proceeds is plotted onto an amplification plot by the SDS software. From this plot a threshold line is set as the level of detection where the fluorescence intensity is greater than any background fluorescence. This line must fall within the exponential phase of detection. The point at which the fluorescence detected in a particular sample crosses this line is known as the cycle threshold (Ct). This value is important for calculating the amount of bacterial DNA in a sample.

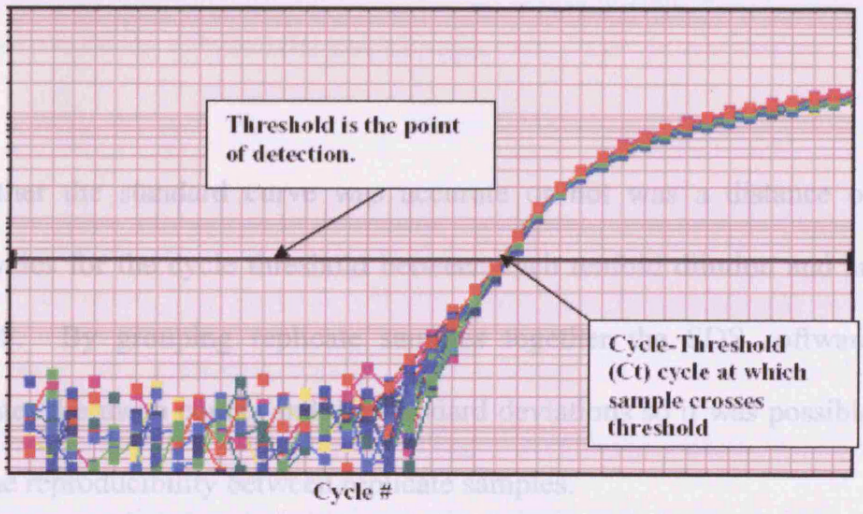

Figure 2.5.1: Typical Amplification plot (from ABI guidelines)

2.5.5.2 Standard curves

To generate a standard curve from DNA extracted from known quantities of bacterial cells this Ct number is plotted against the log number of bacteria (Fig 2.5.2).

Figure 2.5.2: Example of a standard curve generated by SDS software. (from www.inra.fr)

A measure of whether the standard curve was accurate or not was a distance of approximately 3.3 cycles for the cycle threshold between each tenfold dilution and an R_2 value above 0.98. By grouping replicate samples together the SDS software automatically calculates the mean counts and the standard deviations so it was possible to immediately see the reproducibility between replicate samples.

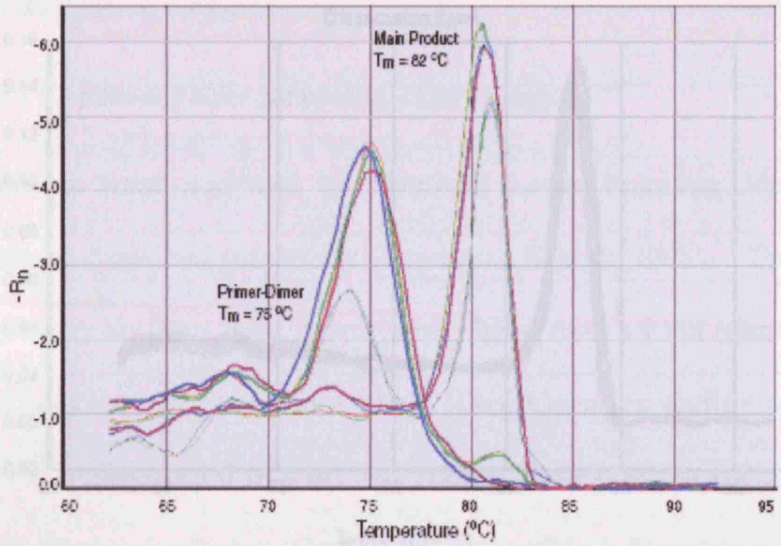

Figure 2.5.3: Typical dissociation curve from a qPCR reaction to detect non-specific amplification products. (From ABI guidelines).

The aim of dissociation curve analysis is to determine the melting temperature (T_m) of the target nucleic acid sequence. This process will also determine if there are any non-specific products as they will characteristically have a lower T_m than the specific product as observed in Fig. 2.5.3. The figure below (Fig. 2.5.4) demonstrates the ideal dissociation curve where no non-specific products are present and a single peak is observed for the target amplification.

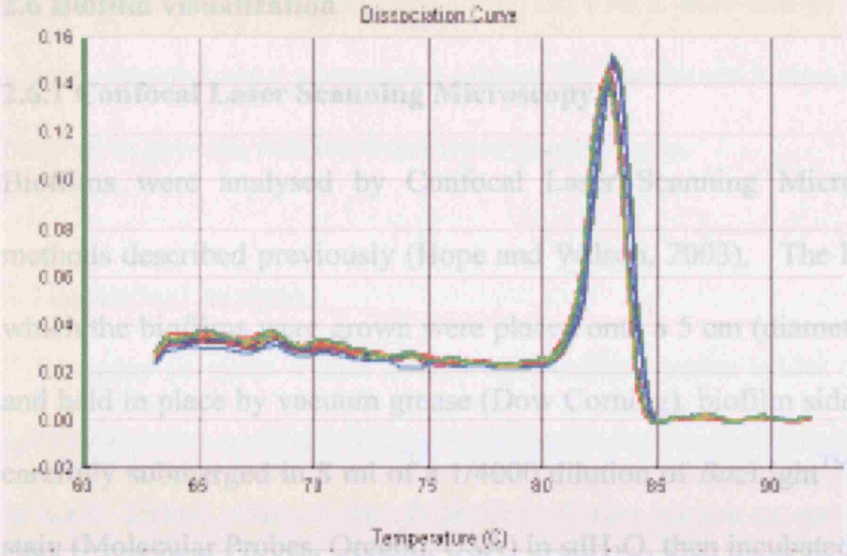

Figure 2.5.4: Ideal dissociation curve. (From ABI guidelines)

2.6 Biofilm visualization

2.6.1 Confocal Laser Scanning Microscopy

Biofilms were analysed by Confocal Laser Scanning Microscopy (CLSM) using methods described previously (Hope and Wilson, 2003). The hydroxyapatite discs on which the biofilms were grown were placed onto a 5 cm (diameter) petri dish (Sarstedt) and held in place by vacuum grease (Dow Corning), biofilm side up. The biofilms were carefully submerged in 8 ml of a 1/4000 dilution of *Bac*Light™ LIVE/DEAD viability stain (Molecular Probes, Oregon, USA) in sdH_2O, then incubated in the dark for 10 min. Replicates were examined with a Radiance 3000 confocal laser-scan head (Biorad GmbH, Jena, Germany) in conjunction with a BX51 stereomicroscope (Olympus UK Ltd, Southall, UK) equipped with a 40 x HCX water immersion dipping lens with a numerical aperture of 0.8 μm. The lasers used were a Helium Neon (543 nm) laser and an Argon (488 nm) laser. The thickness of each confocal optical section was 3 μm. The resulting collections of confocal optical sections were collected by BioRad Lasersharp software as stacks of images (*.PIC) and archived onto optical discs. At each time point three biofilms were examined by CLSM and for each disc at least three different points within the biofilm were observed.

2.6.2 Image analysis

All images (*.PIC files) were analyzed using Image J 1.22d software (The National Institute of Health, USA). Three-dimensional images were created from the live (green) and dead (red) colour channels using the 3-D project tool of Image J then combined to create a single RGB stack using RGB merge, allowing the spatial visualisation of live and dead bacteria within the biofilm structure. To examine the relative intensity of fluorescence for the live and dead channels through each optical section of the biofilms

intensity profiles were generated using the Plot Z-axis tool of Image J. The intensity values obtained were normalized against the maximum image intensity value for each channel to give the relative intensity of fluorescence.

2.7 Statistical analysis

Differences in mean viable counts for biofilms grown under different environmental conditions were analysed using the Student's *t*-test using Microsoft Excel. Differences in mean counts obtained by qPCR for different genera or species for biofilms grown under different environmental conditions were also analysed using the Student's *t*-test. Differences in mean viable counts and differences in mean counts obtained by qPCR for biofilms grown in the presence of different antimicrobial agents compared to controls were also analysed using the Student's *t*-test. For all of the above a significant difference was associated with a *P* value less than 0.05.

CHAPTER 3

Development of an *in vitro* model of the supragingival plaque associated with gingivitis

3.1 Introduction

Periodontal diseases, such as gingivitis and periodontitis, are caused by fluctuations in the oral environment which leads to a change in the microbial composition from one associated with health to one related to disease. Oral streptococci tend to dominate the microbiota of most individuals not suffering from any form of periodontal disease. A key change in the microbiota of supragingival plaque observed by experimental gingivitis studies is the ascendancy of *Actinomyces* spp. and Gram-negative rods at the expense of *Streptococcus* spp. (Moore *et al*, 1987; Moore & Moore, 1994; Syed & Loesche, 1978; Zee *et al*, 1996). *Actinomyces* spp. are initial colonizers of the tooth surface (Marsh & Martin, 1999) and are particularly associated with the accumulation phase of plaque development (Liljemark *et al*, 1993). *A. israelii* is associated with non-bleeding gingivitis, while *A. viscosus* and *A. naeslundii* are associated with bleeding gingivitis (Syed & Loesche, 1978). Gram-negative species indicative of the changes occurring within the plaque environment, such as *F. nucleatum* (Moore *et al*, 1982), *Prevotella* spp. (Jansen & Van der Hoeven, 1997), and *Capnocytophaga* spp. (Mombelli *et al*, 1990), are more frequently isolated from dental plaque associated with gingivitis, due to the development of gradients in oxygen availability and environmental niches that promote their survival.

A key factor associated with gingivitis is increased plaque thickness, allowing the development of gradients in factors such as oxygen potential, pH, and nutrient availability. In addition, protein-based nutrients become available in the form of gingival crevicular fluid (GCF), the flow rate of which increases during the development of gingivitis (Goodson, 2003). Sources of nutrients for plaque bacteria include saliva, GCF, and dietary sources (Wimpenny, 1997) as well as nutrients derived from the

degradation of the extracellular polysaccharides present in the biofilm. However, this nutrient source is only utilised by oral bacteria producing enzymes able to break down these complex macromolecules (Igarashi *et al*, 2004). Changes in these nutritional components are likely to influence plaque composition. Inflammation of the marginal gingiva is important in early supragingival plaque accumulation (Daly & Highfield, 1996), which suggests that inflammatory factors influence bacteria involved in plaque accumulation. Indeed, *Actinomyces* spp. are more frequently isolated from subjects with a strong inflammatory response to gingivitis (Lie *et al*, 1995).

Due to the heterogeneity of plaque composition both between individuals and from different sites within the mouth of an individual (Anderson *et al*, 2002) there is a need to create models of the oral environment in which stable populations can be grown. *In vitro* models of the oral cavity allow for greater control of the environmental factors which contribute to this variation in plaque composition, such as nutrient source, temperature, pH, oxygen availability, and substrata (Wilson, 1999). The CDFF is established as a representative model for dental plaque (McBain *et al*, 2003a; Pratten *et al*, 1998b) producing diverse microbial populations (Pratten *et al*, 2003a) that maintain key oral species at levels similar to those observed *in vivo*. As key parameters can be controlled using this model, changes in plaque composition can be directly linked to specific environmental changes.

Novel approaches to assess changes in the biofilm community as a whole include Community Level Physiological Profiles (CLPP) which have previously been used to assess the microbial community dynamics of soil and water samples and dental plaque microcosms (Anderson *et al*, 2002). This profile is created using BIOLOG plates (96

well microtitre plates, each well containing a different substrate apart from one negative control well) to assess the utilization of sole carbon sources by the bacterial community. The reduction of tetrazolium violet (redox-sensitive dye) to formazan in each well is used as an indicator of substrate utilization by the microbial community as a whole (measured as optical density) if this colour development is greater than that observed in the absence of substrate (the control well). This profile of substrate utilization can be used to create a metabolic fingerprint (Garland, 1997). This technique may be useful in defining *in vitro* biofilms associated with health and gingivitis based on community dynamics.

The aim of this part of the study was to examine the influence of different environmental parameters on oral microcosm biofilms and to emulate the conditions associated with gingivitis in order to instigate changes in the composition of oral biofilms associated with the development of gingivitis.

3.2 Methods

3.2.1 Investigating the influence of different environmental parameters on biofilm development

3.2.1.1 Biofilm production

Microcosm biofilms were generated and genera were enumerated using selective culture techniques as described in Chapter 2 (Section 2.1).

3.2.1.2 Inoculation procedure

Variations in the inoculation procedure were tried in order to maintain the populations observed in the pooled saliva. In order to assess under which conditions species proportions were maintained closest to that observed in pooled saliva, 100 µl of pooled saliva was used to inoculate 50 ml of artificial saliva (the same proportions of pooled saliva to artificial saliva used in the CDFF inoculation procedure) and incubated for 8 hours under aerobic, anaerobic and microaerophilic conditions, under constant agitation. Serial dilutions of pooled saliva and the 8-hour inoculum incubated under different conditions were plated out onto CBA, FAA, MS, CFAT, Rogosa and Veillonella agar to enumerate the total aerobes, total anaerobes, *Streptococcus* spp., *Actinomyces* spp., *Lactobacillus* spp. and *Veillonella* spp. respectively. CBA was incubated aerobically overnight at 37°C and all the other media were incubated anaerobically at 37°C for 4 days.

3.2.1.3 Recess depth

The influence of the depth at which the substrata are recessed below the depth of the scraper blades would have on biofilm growth was also investigated by using two different recess depths, 300 µm (representative of smooth surface plaque thickness,

Main *et al*, 1984) and an increased thickness of 600 μm (more representative of accumulated plaque in interdental regions, Newman & Morgan, 1980). The experiments were carried out in the same fermenter to observe whether an increase in bacterial counts or a change in species proportions could be observed.

3.2.1.4 Substratum

The surfaces for the development of supragingival plaque are the enamel of the teeth and the gingival epithelial cells. As it was not feasible to develop a surface derived of only epithelial cells collagen-coated hydroxyapatite was used as it is an important component of animal tissue structure. Bovine enamel and hydroxyapatite, the mineral component of enamel, were selected as appropriate substitutes to human enamel. Species proportions on the different substrata were examined at several times during biofilm development.

3.2.1.4.1 Collagen coating of hydroxyapatite discs

The maximal binding of collagen to HA has previously been determined as between 20 and 30 μg mg $^{-1}$ HA (Naito & Gibbons, 1988). Each HA disc weighed approximately 100 mg so the amount of collagen to be coated onto each disc was calculated from this weight. The following solutions of Type I collagen (Sigma) were made up in 0.1 % acetic acid for coating of HA discs.

Collagen:Hydroxyapatite Ratio	Solution Concentration
10 μg mg $^{-1}$	**1** mg ml^{-1}
20 μg mg $^{-1}$	**2** mg ml^{-1}
30 μg mg $^{-1}$	**3** mg ml^{-1}

Table 3.2.1: Collagen solutions used to coat HA discs.

To assess the optimum concentration of collagen solution to use to coat the HA discs sterile HA discs were left in the different solutions for 1 hour then left to dry at 37°C for 1 hour. To ensure that collagen was bound to the HA discs they were stained in 0.1% acriflavine (Sigma) which stains collagen green. The optimum concentration to use was determined as 20 μg mg $^{-1}$ (2 mg ml $^{-1}$ solution) which was used to coat HA discs in the CDFF.

To assess the influence of coating of HA discs with collagen on biofilm formation CDFFs were setup and sterilised as described in Chapter 2 (Section 2.1). 2 mg ml $^{-1}$ collagen solution was dropped onto HA discs using a sterile plastic pipette (Sarstedt) via the sampling port. Only half of the HA discs in the CDFF were coated in this way, the rest were left to be used as controls for comparison. The collagen solution was then left to dry on the discs overnight. To ensure that the HA discs had not been contaminated during this procedure 1 pan of collagen coated HA discs was removed at this point and the discs placed into 1 ml of sterile PBS containing 5 glass beads, vortexed for 1 min then the neat solution was plated out onto FAA and CBA and incubated accordingly (described in Chapter 2) to ensure that there was no contamination of the discs. CDFFs were then inoculated and sampled as described in Chapter 2.

3.2.2 Changes in environmental parameters associated with gingivitis

Changes in environmental parameters specifically associated with gingivitis were implemented on dual-species biofilms.

3.2.2.1 Production of dual-species biofilms

Dual-species biofilms of *A. naeslundii* NCTC 10301 and *S. sobrinus* NCTC 12279 were grown in the CDFF as described in Chapter 2 (Section 2.1).

3.2.3 Community Level Physiology Profiling (CLPP)

Changes in dual-species biofilms associated with changing environmental parameters were assessed using CLPP. Substrate utilization analysis was performed with Biolog Gram-positive (GP2) microplates (Biolog Inc. Hayward, CA, U.S.A.), containing 95 different substrates and the colour redox indicator dye, tetrazolium violet. At each time point, biofilms were dispersed by vortexing in 1 ml of GN/GP inoculating fluid (Biolog, Hayward, CA) supplemented with 7.66% sodium thioglycolate (Biolog) with 5 glass beads. A further 19 ml of inoculating fluid was added to this suspension and vortexed. 150 µl of this inoculating fluid was then added to each well of a GP2 microplate. Plates were then wrapped in aluminium foil with a damp paper towel and incubated overnight anaerobically at 37°C. Colour development was measured on a 96-well plate reader (Dynex Technologies, Worthing, UK) at OD_{590} nm, against the control well, which contained no substrate. A well was regarded as positive when it yielded a net OD_{590} greater than 140% of the control (Verschuere *et al*, 1997). To analyse the utilisation patterns of the substrates sixty-five of the ninety-five were grouped into eight functional/structural groups (Table 3.2.2) and the number of positive reactions from each group was calculated.

Table 3.2.2: Carbon substrates used from the 96-well Biolog GP2 microplate.

(From Spratt & Pratten, 2006)

3.2.4 Confocal Laser Scanning microscopy of biofilms

CLSM in conjunction with viability staining was carried out on selected biofilms as described in Chapter 2 (Section 2.6). The procedure for CLSM in conjunction with Gram-staining was identical to the procedure for viability staining except the biofilms were carefully submerged in 8 ml of a 1/4000 dilution of *Bac*Light™ Gram stain (Molecular Probes, Oregon, USA) in sdH_2O, then incubated in the dark for 10 min.

3.3 Results

3.3.1 Inoculation procedure

The composition of the saliva and the inoculum was assessed using selective culture media for key genera and total bacteria. The inoculum was incubated overnight under aerobic, anaerobic and microaerophilic conditions in artificial saliva to assess under which conditions species proportions would be maintained closest to the composition of saliva. *Streptococcus* spp. numbers were the same under all conditions whilst *Actinomyces* spp. numbers were reduced under aerobic conditions (Fig. 3.3.1). *Lactobacillus* spp. numbers were significantly reduced (P <0.05) under all conditions but were higher under anaerobic and microaerophilic conditions. *Veillonella* spp. numbers were maintained under anaerobic and microaerophilic conditions but were significantly reduced (P <0.05) under aerobic conditions.

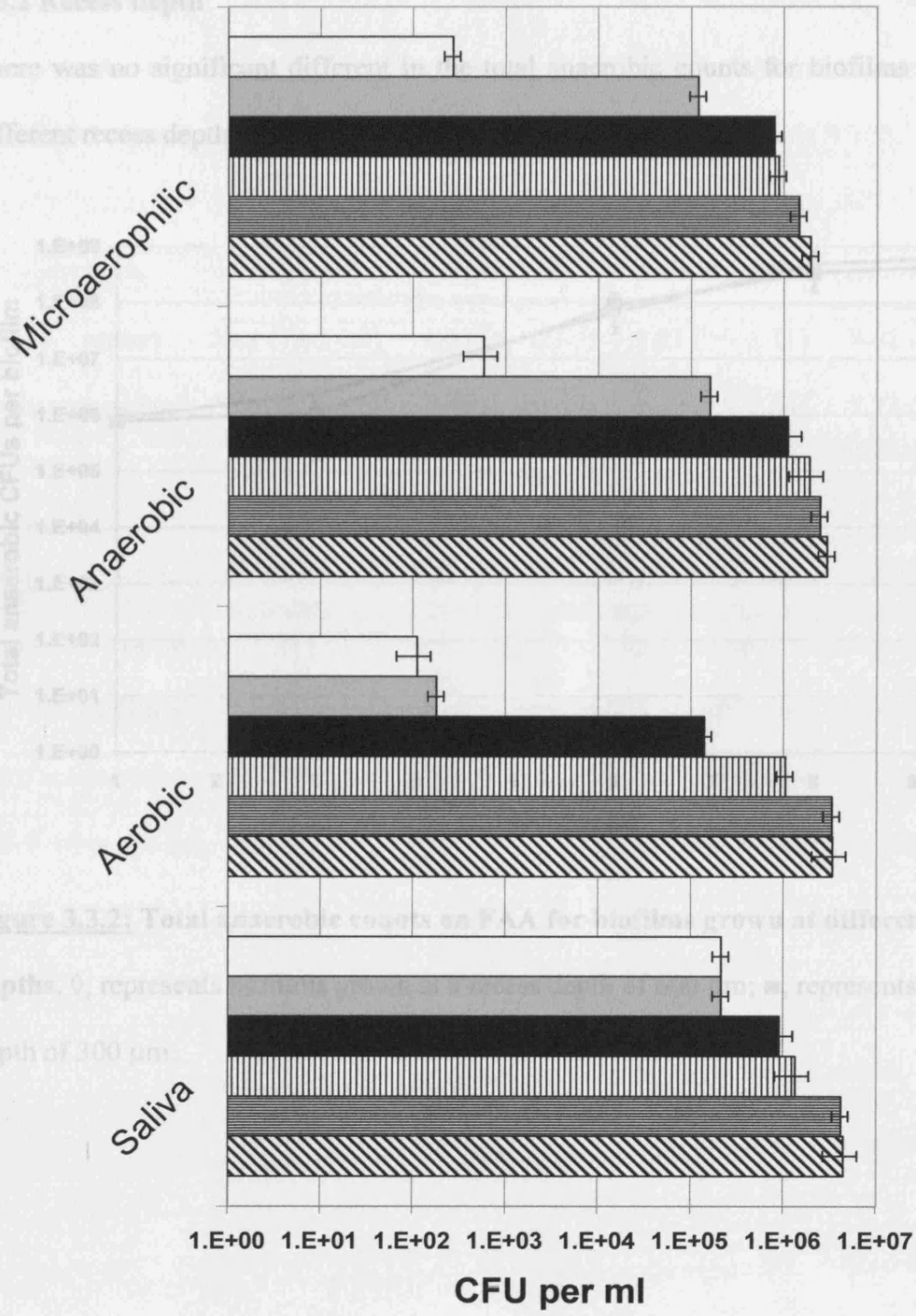

Figure 3.3.1: Composition of saliva and inoculum under different conditions. □, represents *Lactobacillus* spp.; ■, represents *Veillonella* spp.; ■, represents *Actinomyces* spp.; ▥, represents *Streptococcus* spp; ▤ , represents total aerobes; ▨, represents total anaerobes. Error bars represent standard deviations ($n = 3$).

3.3.2 Recess depth

There was no significant different in the total anaerobic counts for biofilms grown at different recess depths in the same fermenter (Fig. 3.3.2).

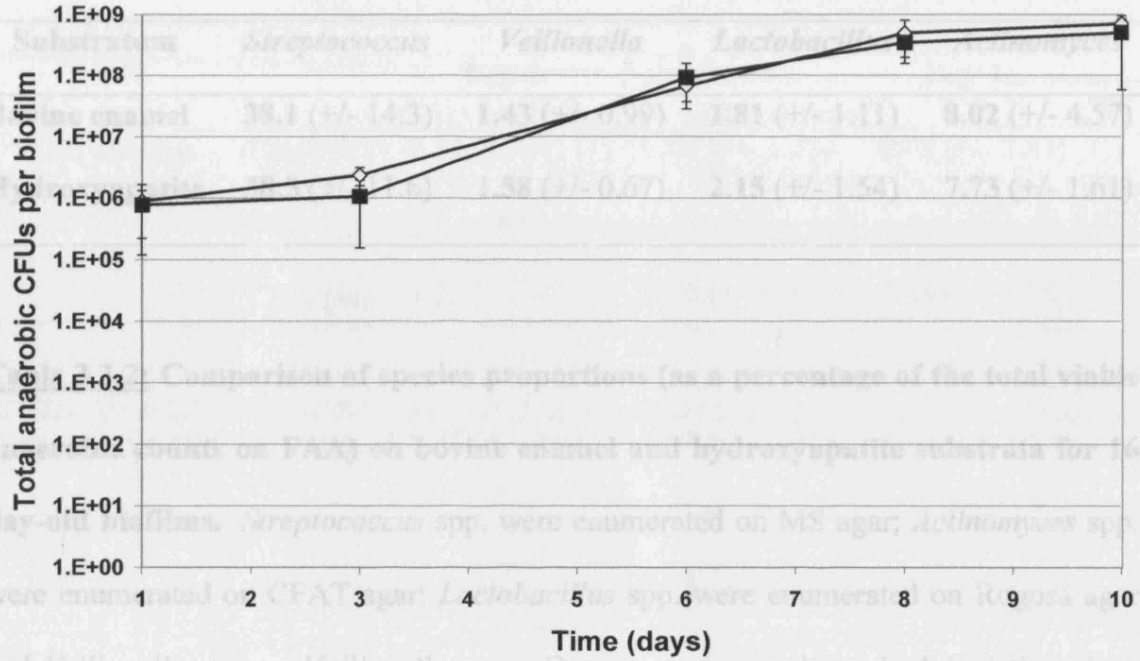

Figure 3.3.2: Total anaerobic counts on FAA for biofilms grown at different recess depths. ◊, represents biofilms grown at a recess depth of 600 μm; ■, represents a recess depth of 300 μm.

3.3.3 Substratum

There appeared to be no difference in the species composition of microcosm biofilms developed on bovine enamel or HA discs in the same fermenter (Table 3.3.1).

Substratum	*Streptococcus*	*Veillonella*	*Lactobacillus*	*Actinomyces*
Bovine enamel	**38.1** (+/- 14.3)	**1.43** (+/- 0.99)	**1.81** (+/- 1.11)	**8.02** (+/- 4.57)
Hydroxyapatite	**38.5** (+/- 11.6)	**1.58** (+/- 0.67)	**2.15** (+/- 1.54)	**7.73** (+/- 1.61)

Table 3.3.2: Comparison of species proportions (as a percentage of the total viable anaerobic counts on FAA) on bovine enamel and hydroxyapatite substrata for 16 day-old biofilms. *Streptococcus* spp. were enumerated on MS agar; *Actinomyces* spp. were enumerated on CFAT agar; *Lactobacillus* spp. were enumerated on Rogosa agar and *Veillonella* spp. on Veillonella agar. Data are means and standard deviations (n = 3).

3.3.4 Collagen coating of hydroxyapatite discs

3.3.4.1 Cultural analysis

There was no difference in the total aerobic and anaerobic counts determined by culture for biofilms grown on normal HA discs or collagen-coated HA discs (Table 3.3.3).

		Day 1	Day 7	Day 16
Total aerobes	**Control**	**4.55** (+/- 3.11) x 10^6	**6.63** (+/- 1.26) x 10^8	**6.88** (+/- 3.83) x 10^8
	Collagen	**7.23** (+/- 4.55) x 10^6	**6.50** (+/- 0.88) x 10^8	**7.25** (+/- 4.55) x 10^8
Total anaerobes	**Control**	**6.41** (+/- 2.59) x 10^6	**6.90** (+/- 3.27) x 10^8	**8.02** (+/- 3.17) x 10^8
	Collagen	**6.43** (+/- 0.98) x 10^6	**6.75** (+/- 1.16) x 10^8	**8.38** (+/- 4.11) x 10^8

Table 3.3.3: Total aerobes and anaerobes in biofilms grown on HA or collagen-coated HA. Data are means and standard deviations (n = 4).

The total counts for different genera determined by culture on selective media showed no difference in the composition of 7-day-old biofilms grown on standard HA discs or collagen-coated HA discs (Fig. 3.3.3).

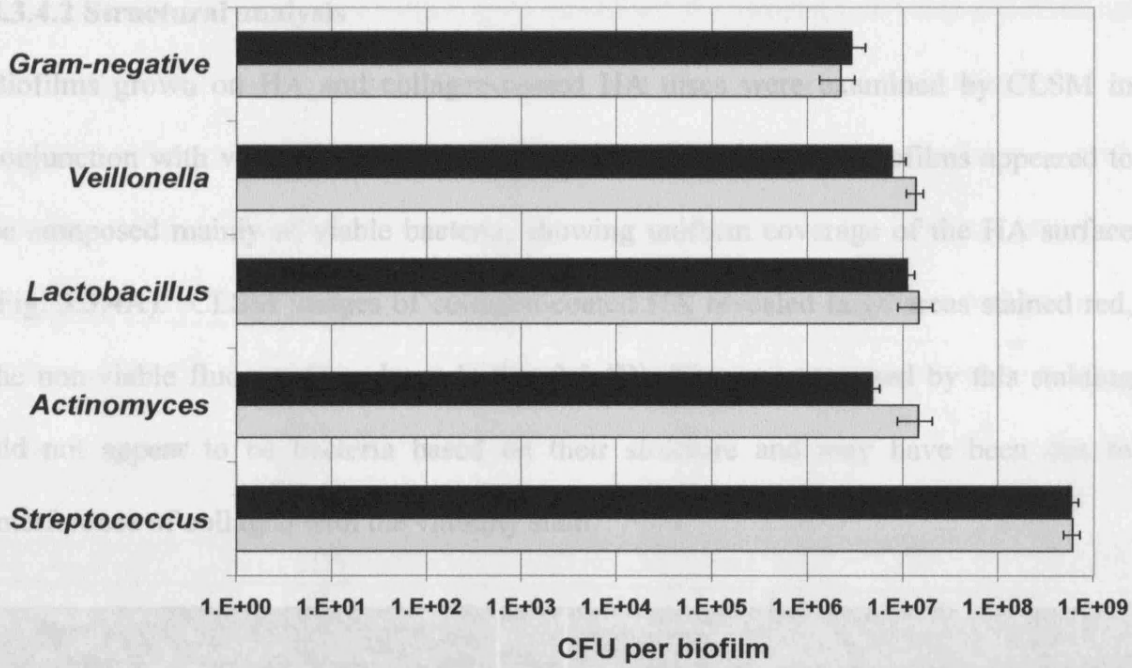

Figure 3.3.3: Species counts determined by culture for 7-day-old biofilms grown on HA or collagen-coated HA. ■, represents biofilms grown on HA; ■, represents biofilms grown on collagen-coated HA. Error bars represent standard deviations (n = 4).

3.3.4.2 Structural analysis

Biofilms grown on HA and collagen-coated HA discs were examined by CLSM in conjunction with viability staining and Gram-staining. 1-day-old biofilms appeared to be composed mainly of viable bacteria, showing uniform coverage of the HA surface (Fig. 3.3.4A). CLSM images of collagen-coated HA revealed large areas stained red, the non-viable fluorescence channel (Fig. 3.3.4B). The areas covered by this staining did not appear to be bacteria based on their structure and may have been due to interference of collagen with the viability stain.

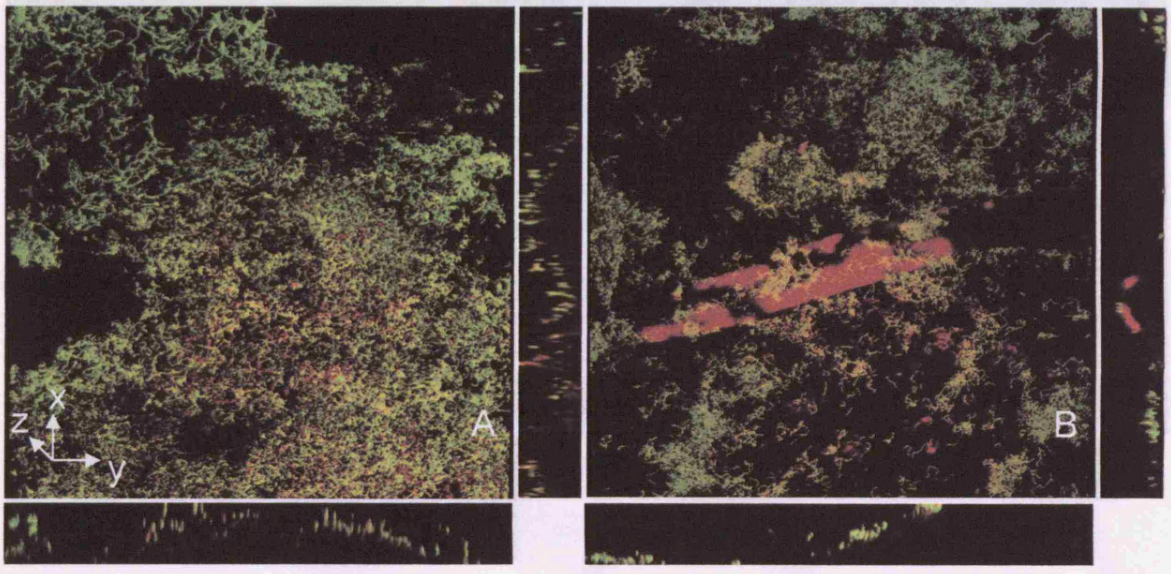

Figure 3.3.4: CLSM images (300 by 300 µm) showing viability of 1-day-old microcosm biofilms. A, represents biofilms grown on HA; B, represent biofilms grown on collagen-coated HA. Green represents viable bacteria, red represents non-viable bacteria. Images are *xy* sections taken through layers (1-3 µm) of the biofilm merged to create a 3D projection of the *z* axis. Arrows indicate directions of the *x*, *y* and *z* planes. Side panels are sagittal (*xz*) images of the biofilm taken from specific points along the *xy* axis.

Gram-staining in conjunction with CLSM visualised the structure of 1-day-old biofilms differently from viability staining (Fig 3.3.5). Coverage of the HA surface appeared to be less uniform with bacteria forming clumps rather than the layers observed with viability staining. This effect appeared to be more pronounced for biofilms grown on collagen-coated HA and again this effect may have been due to the interference of collagen with the stain. The biofilms appeared to be composed mainly of Gram-positive bacteria as mainly red fluorescence was detected.

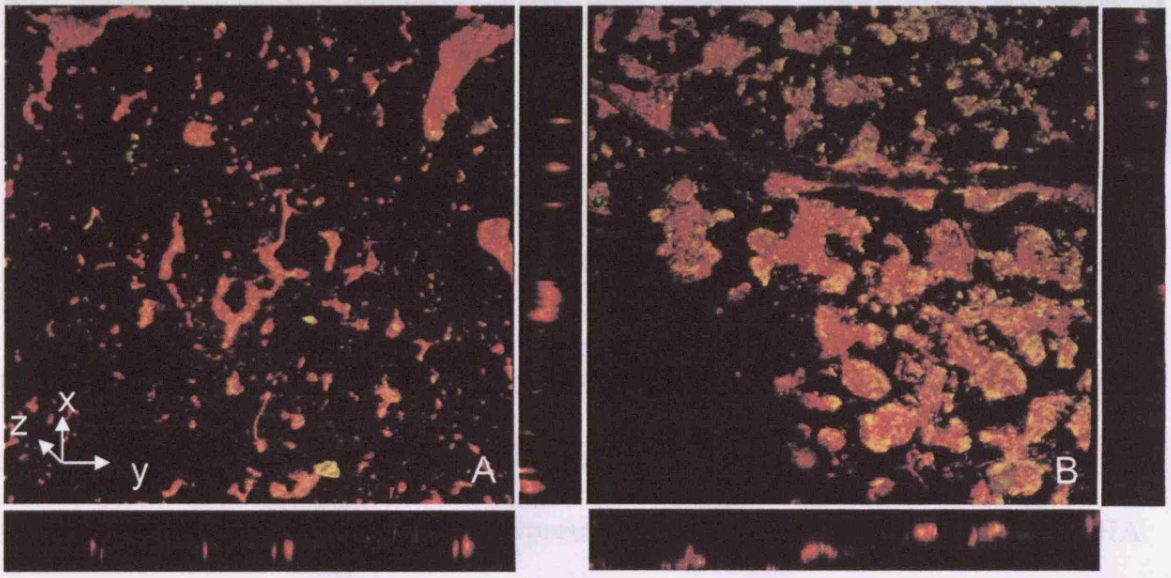

Figure 3.3.5: CLSM images (300 by 300 μm) showing bacteria Gram-positive and Gram-negative bacteria in 1-day-old microcosm biofilms. A, represents biofilms grown on HA; B, represent biofilms grown on collagen-coated HA. Green represents Gram-negative bacteria, red represents Gram-positive bacteria. Images are *xy* sections taken through layers (1-3 μm) of the biofilm merged to create a 3D projection of the *z* axis. Arrows indicate directions of the *x*, *y* and *z* planes. Side panels are sagittal (*xz*) images of the biofilm taken from specific points along the *xy* axis.

For 7-day-old biofilms comparisons of the structure by viability staining revealed bacteria were forming aggregates with large visible voids (Fig. 3.3.6). Biofilms grown on collagen coated HA discs demonstrated large aggregates of non-viable bacteria (Fig. 3.3.6B).

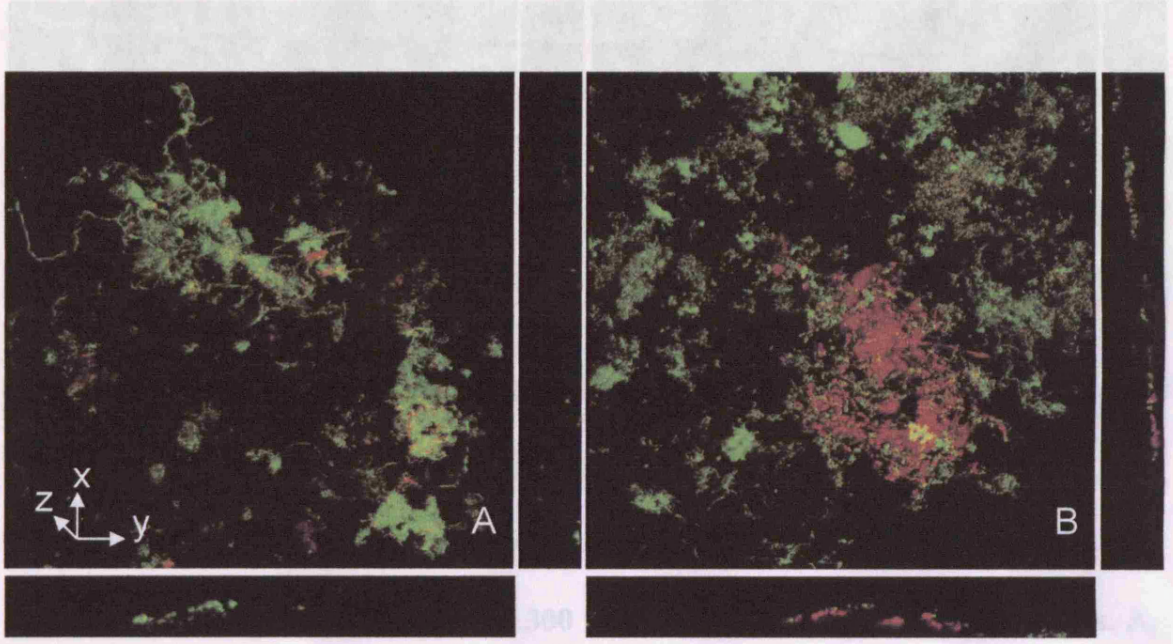

Figure 3.3.6: CLSM images (300 by 300 μm) of 7-day-old microcosm biofilms. A, represents biofilms grown on HA; B, represent biofilms grown on collagen-coated HA. Green represents viable bacteria, red represents non-viable bacteria. Images are *xy* sections taken through layers (1-3 μm) of the biofilm merged to create a 3D projection of the *z* axis. Arrows indicate directions of the *x*, *y* and *z* planes. Side panels are sagittal (*xz*) images of the biofilm taken from specific points along the *xy* axis.

Again, the structure appeared different with Gram-staining (Fig. 3.3.7), with fewer visible voids present, especially for biofilms grown on collagen-coated HA (Fig. 3.3.7B). In contrast to 1-day-old biofilms, Gram-negative bacteria appeared to dominate 7-day-old communities grown both on HA and collagen-coated HA discs.

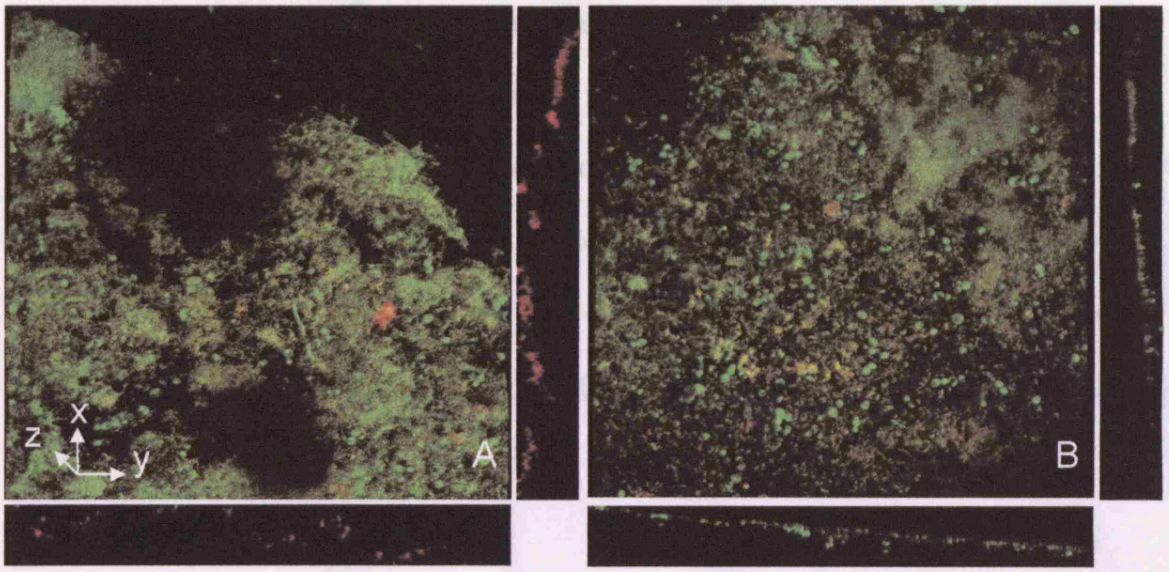

Figure 3.3.7: CLSM images (300 by 300 μm) of 7-day-old microcosm biofilms. A, represents biofilms grown on HA; B, represent biofilms grown on collagen-coated HA. Green represents Gram-negative bacteria, red represents Gram-positive bacteria. Images are *xy* sections taken through layers (1-3 μm) of the biofilm merged to create a 3D projection of the *z* axis. Arrows indicate directions of the *x*, *y* and *z* planes. Side panels are sagittal (*xz*) images of the biofilm taken from specific points along the *xy* axis.

3.3.5 Dual-species biofilms

3.3.5.1 Cultural analysis

In dual-species biofilms grown aerobically and fed with artificial saliva, *S. sobrinus* was the dominant organism throughout, achieving a stable population after 7 days, with viable counts of 5.4×10^7 CFU per biofilm. In contrast, *Actinomyces* spp. reached a maximum viable count of 5.7×10^5 CFU per biofilm, although the counts were also stable by day 7 (Fig. 3.3.8). With the addition of artificial GCF and microaerophilic gas into the system, *A. naeslundii* became the dominant organism, with counts of 3.5×10^8 CFU per biofilm, representing a significant increase ($P < 0.05$) in numbers. *S. sobrinus* counts were significantly reduced ($P < 0.001$) to a minimum of 1.6×10^6 CFU per biofilm.

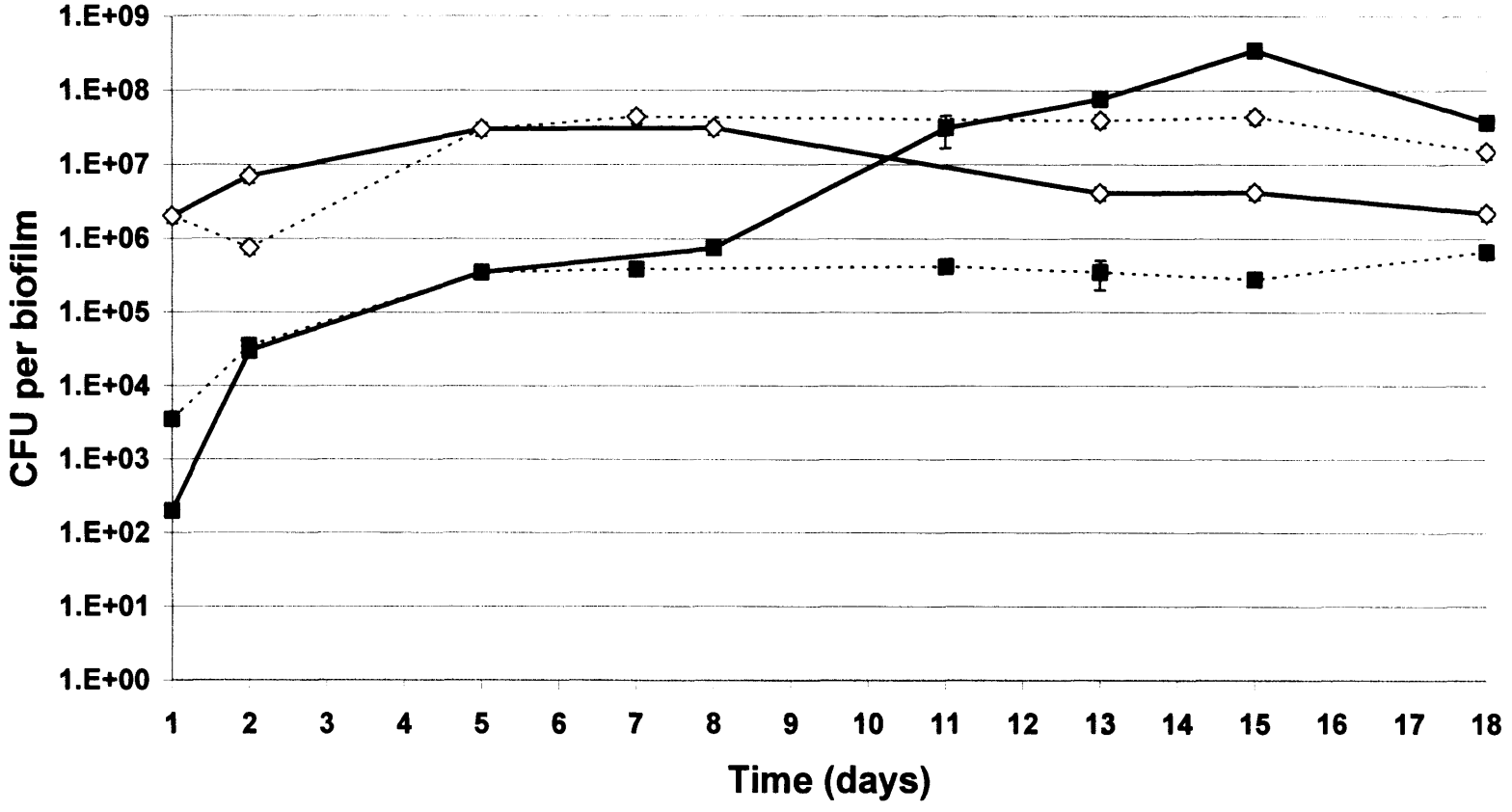

Figure 3.3.8: Species counts in dual-species biofilms.■, *A. naeslundii*;◊, *S. sobrinus*. Solid lines represent experiments when artificial GCF addition and a switch to microaerophilic conditions were commenced on day 8. Dashed lines represent experiments in which no change in the conditions was implemented. Error bars represent the standard deviations ($n = 4$).

3.3.5.2 Structural analysis

Examination of dual-species biofilms by CLSM (Fig. 3.3.9) revealed that after the addition of artificial GCF and microaerophilic gas biofilms demonstrated a more tightly packed structure with non-viable bacteria being more prominent in all layers of the biofilm than before addition.

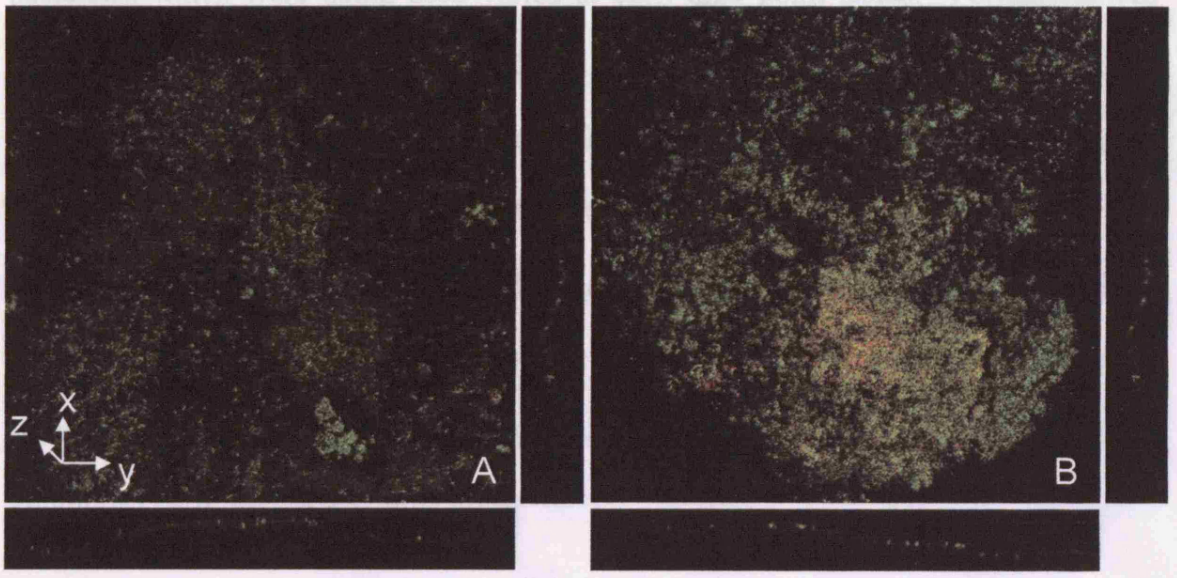

Figure 3.3.9: 3D CLSM images (300 by 300 μm) of dual-species biofilms. A, represents 8-day-old biofilms before the addition of artificial GCF and microaerophilic gas; B, represents 15-day-old biofilms after addition. Green represents viable bacteria, and red represents non-viable bacteria. Images are *xy* sections taken through layers (1-3 μm) of the biofilm merged to create a 3D projection of the *z* axis. Arrows indicate directions of the *x*, *y* and *z* planes. Side panels are sagittal (*xz*) images of the biofilm taken from specific points along the *xy* axis.

3.3.5.3 Community analysis

Planktonic overnight cultures of *A. naeslundii* and *S. sobrinus* showed a greater degree of metabolic activity, with a greater number of carbon sources utilized, than biofilms sampled at any time point (Table 3.3.4). Biofilms utilized the greatest number of substrates initially and then after conditions emulating gingivitis were instigated. The substrates which were being used varied at each time point tested. Polysaccharides (Group 1) appeared to be most utilised in initial communities, under conditions emulating health, as were sugar alcohols (Group 4). The use of monomers (Group 2) appeared to be reduced after 18 days, whilst the use of carboxylic acids (Group 5) was highest at this point. Amino acids did not appear to be utilized much in communities grown under either condition. Nucleosides (Group7) and sugar phosphates (Group 8) were both utilised on day 3, no longer utilised on day 6 but then utilised again once gingivitis conditions were commenced.

	Substrate Group [a]								No. of carbon sources utilized [b]
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	
A. naeslundii [c]	5	12	9	5	11	1	7	4	54
S. sobrinus [c]	5	10	7	0	5	5	6	2	40
Day 3 [d]	4	3	4	3	2	2	2	1	21
Day 6 [d]	3	4	3	1	3	1	0	0	15
Day 10 [e]	0	4	1	0	3	1	3	1	13
Day 18 [e]	2	1	4	1	6	2	3	3	22

Table 3.3.4: Changes in the microbial community over time as assessed by the number of Gram-positive wells per substrate group and the total number of carbon sources utilised (*a*, For substrate groups see Table 3.2.2; *b*, total 65; *c*, overnight cultures grown in BHI; *d*, under conditions emulating health; *e*, under conditions emulating gingivitis after day 7).

Duplicate BIOLOG plates of biofilm communities removed at several time points, before and after artificial GCF and microaerophilic addition, were analysed to examine whether profiles differed in each state. The duplicate profiles generated from biofilms sampled from the same time point were highly divergent making it difficult to make any comparisons. To ensure this was not due to individual biofilms sampled at the same time point being highly divergent, duplicate plates of simple overnight cultures were also compared (Table 3.3.5). These also did not show high levels of similarity, indicating that very small perturbations in inoculum composition could result in divergent profiles so this approach was not continued in subsequent CDFF experiments.

Interestingly, pure cultures of *A. naeslundii* and *S. sobrinus* showed higher levels of substrate utilisation than mixed cultures (Table 3.3.5).

Inoculum	Similarity (%)	Positive reactions
Actinomyces naeslundii	77.9	51
Streptococcus sobrinus	63.2	69.5
Mixed overnight culture		
+ CO_2 (in brain heart infusion broth)	74.7	40.5
Aerobic conditions (in artificial saliva)	74.7	39
Anaerobic conditions (in artificial saliva)	73.7	38

Table 3.3.5: Comparison of reactions between duplicate BIOLOG plates. Similarity is the percentage of identical reactions between duplicate BIOLOG plates from the same condition. The positive reactions represent the utilisation of a particular substrate (all 95 substrates included).

3.4 Discussion

3.4.1 Influence of environmental parameters

The inoculation procedures investigated revealed that under aerobic conditions the numbers of key genera such as *Lactobacillus* and *Veillonella* spp. were significantly reduced. This is unsurprising as a significant portion of the bacteria present in saliva are strict anaerobes (Evaldson *et al*, 1982) with anaerobes outnumbering aerobes 10:1. Under microaerophilic and anaerobic conditions these species were maintained in higher numbers. Microaerophilic rather than anaerobic conditions were selected for use in subsequent experiments as initial colonisers of the tooth are mainly facultative and aerobic species (Li *et al*, 2004; Diaz *et al*, 2006), with strict anaerobes becoming more significant members of the community as oral biofilms mature. The use of different recess depths had no effect on the total numbers of bacteria, however the increased recess depth of 600 μm was used for all subsequent experiments as it could encourage increased retention of nutrients and a more anaerobic environment. The use of bovine enamel or HA discs as substrata also had no effect on biofilm formation and therefore HA discs were selected for subsequent use as they are easier to obtain and re-use without damage. Coating of HA discs with collagen also appeared to have no effect on total bacterial numbers or species composition so this process was not repeated for further experiments as the collagen-coating procedure increased the opportunity for fermenters to become contaminated before inoculation and the establishment of a stable biofilm, without demonstrating any appreciable difference in biofilm composition or structure.

3.4.2 Methods used to analyse biofilms

The aim of using CLSM to visualize the spatial arrangement of these biofilms was to confirm the appearance of structures associated with supragingival plaque. The images produced for both the dual-species and microcosm biofilms showed an increase in proportions of dead bacteria within the biofilm structure when conditions were changed to favour gingivitis plaque development and the emergence of a more tightly packed structure. Key structural features which have previously been observed in supragingival plaque such as voids, channels and filaments were seen, indicating that the plaque developed in the CDFF was comparable to *in vivo* plaque (Guggenheim *et al*, 2001b; Thurneer *et al*, 2004).

The use of Gram-staining in conjunction with CLSM revealed some interesting structures in the biofilms that were not observed with viability staining. More mature biofilms tended to be dominated by Gram-negative bacteria. However, it was also observed that some regions of the biofilm were stained identically with both the Gram-positive and Gram-negative stain making it impossible to determine which Gram reaction was the correct one. According to the manufacturers instructions non-viable bacteria may not display the correct Gram reaction. As non-viable bacteria account for significant portion of oral biofilms, the results of Gram-staining in conjunction with CLSM could not be used to make any reliable conclusions on biofilm structure and composition. Other Gram-stains are currently available which can distinguish between viable and non-viable bacteria and Gram reaction at the same time but these require a confocal microscope with three separate laser channels which was unavailable for this study and so this approach was not pursued in further studies.

Analysis of dual-species biofilms by CLPP revealed that with the introduction of a new nutrient source (artificial GCF) and microaerophilic conditions, different patterns of substrate utilisation were observed. The highest levels of utilisation of carboxylic acids, nucleosides and sugar phosphates were observed with this change in conditions. Interestingly, pure cultures of *A. naeslundii* were able to utilise these substrates more than pure cultures of *S. sobrinus* which may have reflected the increased numbers of *A. naeslundii* detected by culture techniques after the induction of gingivitis conditions.

However, the CLPP profiles obtained from the dual-species biofilms showed high levels of variability between replicate plates and therefore it was difficult to accurately characterise the differences in the profiles obtained before and after the induction of gingivitis conditions. Also, replicate plates of pure cultures of *A. naeslundii* and *S. sobrinus* showed similar levels of variability. Garland, (1997) suggested that differences in inoculum density (which are reflected by the overall rate of colour development) may result in highly divergent profiles which could have accounted for differences seen with biofilm profiles. However, with pure cultures the inoculum density was equal for both plates and the differences observed must be due to other factors. Possible explanations why this technique has worked with soil communities but not for this study are that soil represents a low nutrient environment while dental plaque communities are more nutrient rich and diverse.

The results of previous work done using this technique on dental plaque microcosms (Anderson *et al,* 2002) are not directly comparable to this study as techniques and conditions for biofilm growth were different, particularly with the addition of sucrose to the growth medium. This technique has been used previously to assess microcosm

plaque communities developed in the CDFF (Spratt & Pratten, 2006) and demonstrated that the communities sampled at different time points were distinct, becoming more diverse over time. Much higher levels of substrate utilisation were observed than in this study but as these were microcosm communities, with a greater variety of species present, this difference is unsurprising. Due to the heterogeneous nature of biofilms grown in the CDFF slight differences in composition may result in highly variable profiles which would inhibit producing a unique profile for health or gingivitis communities. The best application for CLPP may be to monitor changes in an individual biofilm community as it evolves, not to compare separate biofilm communities.

3.4.3 Conditions emulating gingivitis

The key nutrient sources for plaque at gingival margins are saliva and gingival crevicular fluid (Rudiger *et al*, 2002). Thus, the addition artificial GCF to the artificial saliva nutrient source was representative of nutritional changes that occur during gingivitis. The main purpose of the dual-species biofilm was to examine the relationship between *A. naeslundii and S. sobrinus* under conditions emulating those that develop during gingivitis. The dual-species model has demonstrated that a controllable and reproducible population shift could be achieved with an ascendancy of *A. naeslundii* correlating with the induction of gingivitis conditions.

3.4.4 Conclusions

The results of this chapter have helped determine the influence of individual environmental parameters on biofilm development in order to define the optimum conditions required for the production of biofilms emulating those that develop during gingivitis. In the dual-species model it was possible to reproducibly instigate a shift in the microbial community by emulating environmental changes associated with gingivitis. The next chapter will describe the influence of these conditions on microcosm biofilms.

CHAPTER 4

Characterisation of microcosm dental plaque associated with health and disease

4.1 Introduction

The supragingival plaque which develops during gingivitis has been shown, by experimental gingivitis studies, to be distinct from healthy plaque (Loesche and Syed, 1978; Syed and Loesche, 1978; Lie *et al,* 1995, Zee *et al,* 1996). One well characterised difference is an increase in species richness as gingivitis develops (Loe *et al*, 1965; Loesche and Syed, 1978). The previous chapter described the development of an *in vitro* model for the growth of supragingival plaque associated with gingivitis. This chapter will describe the characterisation of changes in key species or genera in communities developed in this model using selective culture techniques. The species richness in these communities will be characterized by using 16S rRNA sequencing to identify cultured isolates. Biofilm architecture and the observation of any structural changes as biofilms develop will be characterized using confocal laser scanning microscopy.

4.2 Methods

4.2.1 Microcosm biofilms

The fermenters were set up, inoculated and maintained under conditions associated with health and gingivitis as described in Chapter 2 (Section 2.1). Biofilm samples from different time points and different growth conditions were examined using the techniques described below.

4.2.2 Cultural analysis

Analysis of the cultivable bacteria was carried out on selective and non-selective media as described in Chapter 2 (Section 2.2).

4.2.3 Identification of cultivable species by 16S rRNA PCR

The species isolated by cultural techniques were identified to the species level by amplification and sequencing of the 16S rRNA gene as described in Chapter 2 (Section 2.3).

4.2.4 Confocal Laser Scanning Microscopy (CLSM)

CLSM analysis was carried out on biofilms sampled at different time points and from experiments carried out under different conditions using methods described in Chapter 2 (Section 2.6).

4.3 Results

4.3.1 Cultural analysis

4.3.1.1 Aerobic and anaerobic viable counts

The viable counts obtained for the total aerobic and anaerobic species were very similar for most time points tested (Fig. 4.3.1), however, the total anaerobic count was usually higher.

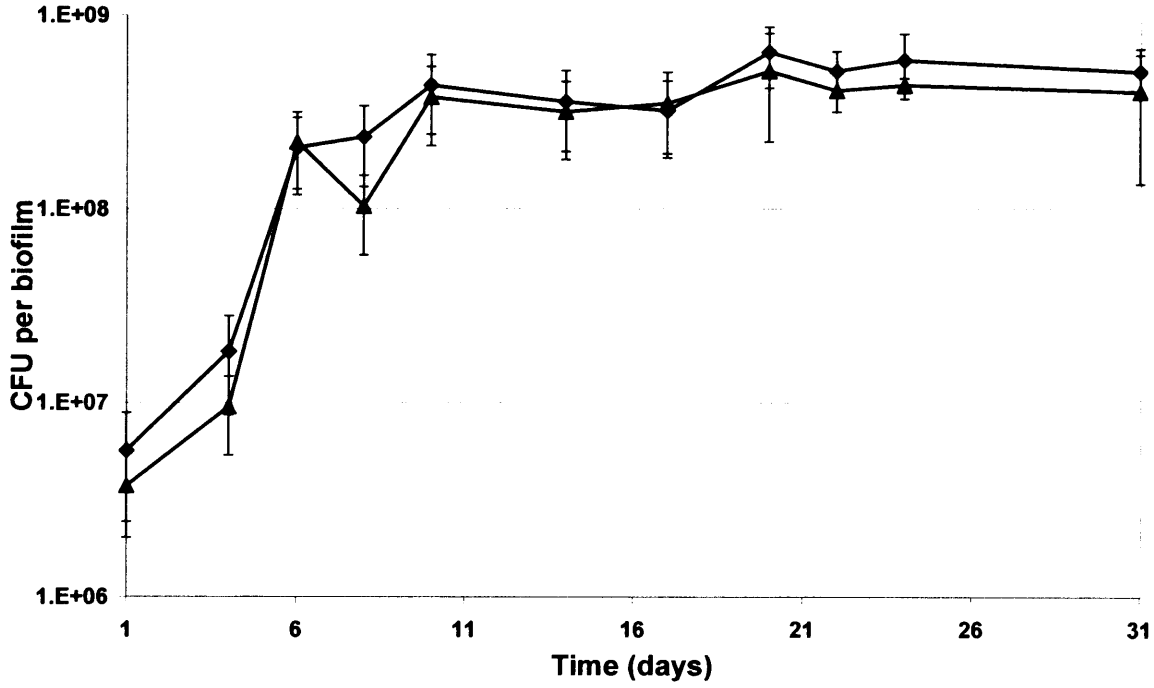

Figure 4.3.1: Total aerobic and anaerobic viable counts for microcosm communities. ♦ represents total anaerobic viable counts obtained on FAA; ▲ represents total aerobic viable counts obtained on CBA. Error bars represent standard deviations (n = 6).

Under conditions emulating health the total aerobic and anaerobic viable counts were very similar (Table 4.3.1). Under conditions emulating gingivitis the total anaerobic and

aerobic viable counts appeared to increase, but looking at the variation in total counts at different time points (Fig. 4.3.1) this increase was not significant.

	CFU per biofilm	
	Health	**Gingivitis**
Total anaerobes	**2.82** (+/- 1.23) x 10^8	**5.11** (+/- 1.64) x 10^8
Total aerobes	**2.13** (+/- 1.29) x 10^8	**3.90** (+/- 0.90) x 10^8

Table 4.3.1: Total aerobic and anaerobic viable counts under conditions emulating health or gingivitis. Data are means +/- standard deviations (n = 10) and were pooled from 4 CDFF experiments.

4.3.1.2 Selective media

In microcosm biofilms, species proportions were stable after approximately 7 days, with *Streptococcus* spp. counts reaching 3.4 x 10^8 CFU per biofilm and *Actinomyces* spp. reaching 1.0 x 10^7 CFU per biofilm. After the addition of artificial GCF and microaerophilic gas, *Actinomyces* spp. became the dominant cultivable genera (Fig. 4.3.2), with total numbers reaching 5.0 x 10^8 CFU per biofilm, representing a significant increase ($P < 0.001$). This was accompanied by a significant decrease ($P < 0.001$) in *Streptococcus* spp. counts to 7.6 x 10^6 CFU per biofilm. In experiments where there was no addition of microaerophilic gas (Fig. 4.3.3) and only artificial GCF was added *Actinomyces* spp. still increased but at a slower rate. For example, after 4 days of artificial GCF plus microaerophilic gas addition *Actinomyces* spp. counts showed a significant ($P < 0.01$) increase (Fig. 4.3.2), whilst in experiments when only artificial GCF was pumped in 9 days of addition was necessary to see a significant increase ($P <$

0.001) in *Actinomyces* spp. counts (Fig. 4.3.3). Similarly, the reduction in *Streptococcus* spp. counts was also much less dramatic, significant reduction ($P <$ 0.001) only being observed after 9 days of artificial GCF addition.

During further experiments, when GCF and microaerophilic gas addition were ceased, *Streptococcus* numbers increased to levels seen before the addition (Fig. 4.3.4). The increase in *Actinomyces* spp. and decrease in *Streptococcus* spp. always corresponded to the point where environmental conditions were changed (Fig. 4.3.4).

For *Veillonella* and *Lactobacillus* spp. after the initial increase during biofilm development numbers appeared to stabilize after 7 days and were then unaffected by altering the environmental conditions (Figs. 4.3.2, 4.3.3 and 4.3.4).

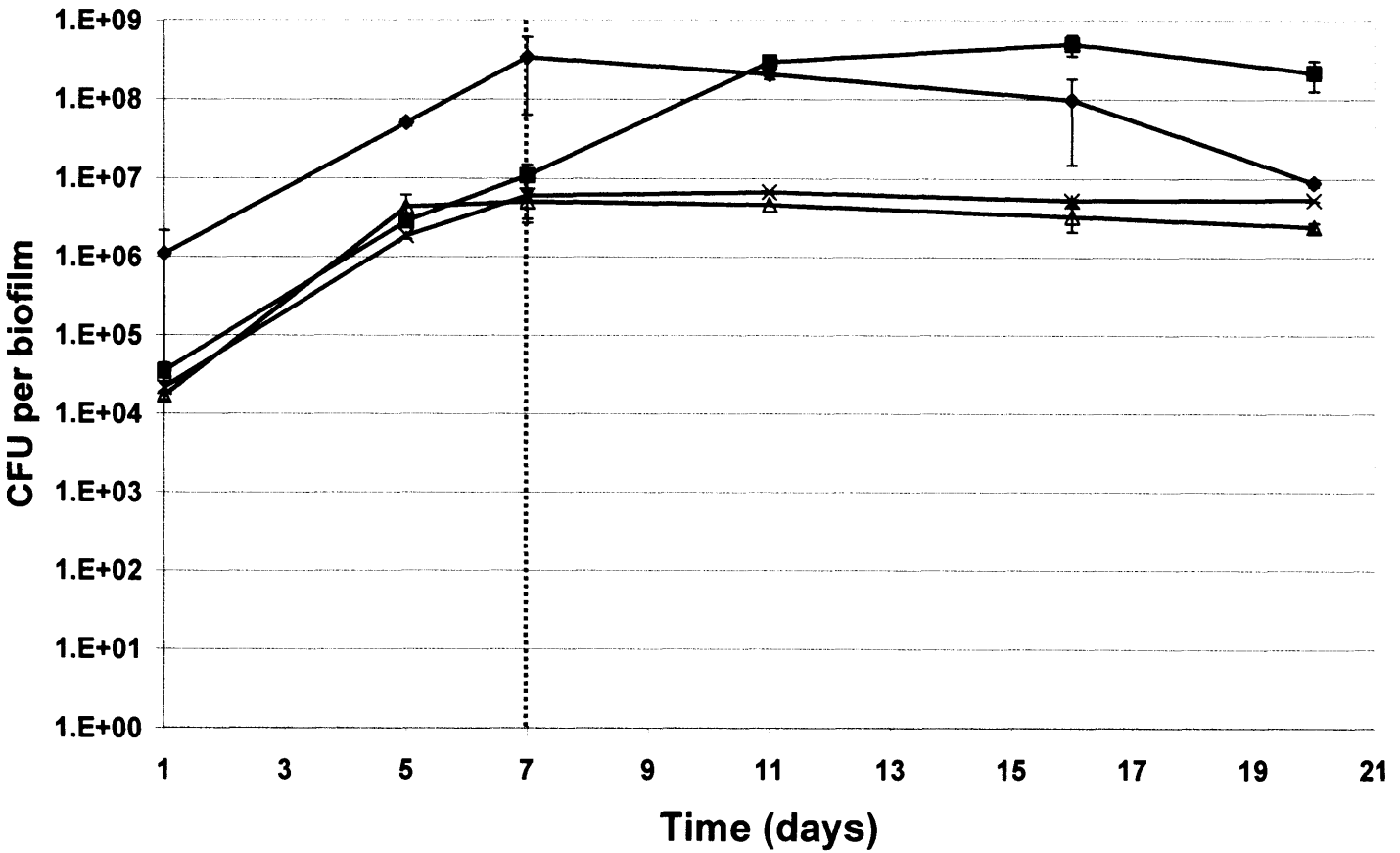

Figure 4.3.2: Viable counts from microcosm biofilms under gingivitis conditions (addition of artificial GCF and microaerophilic gas) from day 7 onwards. ■, *Actinomyces* spp.; ♦, *Streptococcus* spp.; Δ, *Veillonella* spp.; x, *Lactobacillus* spp. Error bars represent the standard deviations ($n = 8$).

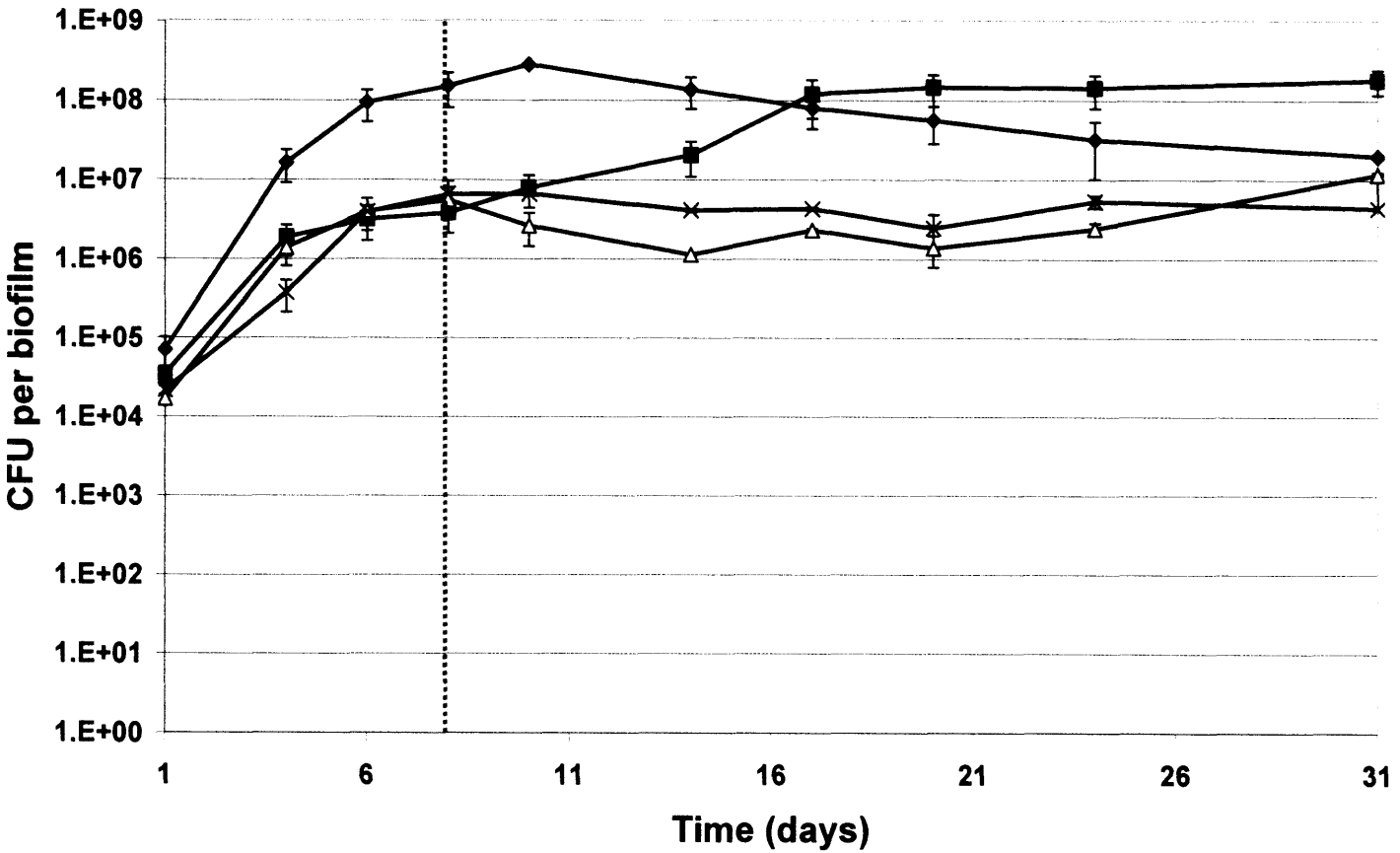

Figure 4.3.3: Viable counts from microcosm biofilms when artificial GCF was added from day 7 onwards. ■, *Actinomyces* spp.; ♦, *Streptococcus* spp.; Δ, *Veillonella* spp.; **x**, *Lactobacillus* spp. Error bars represent the standard deviations (n = 5).

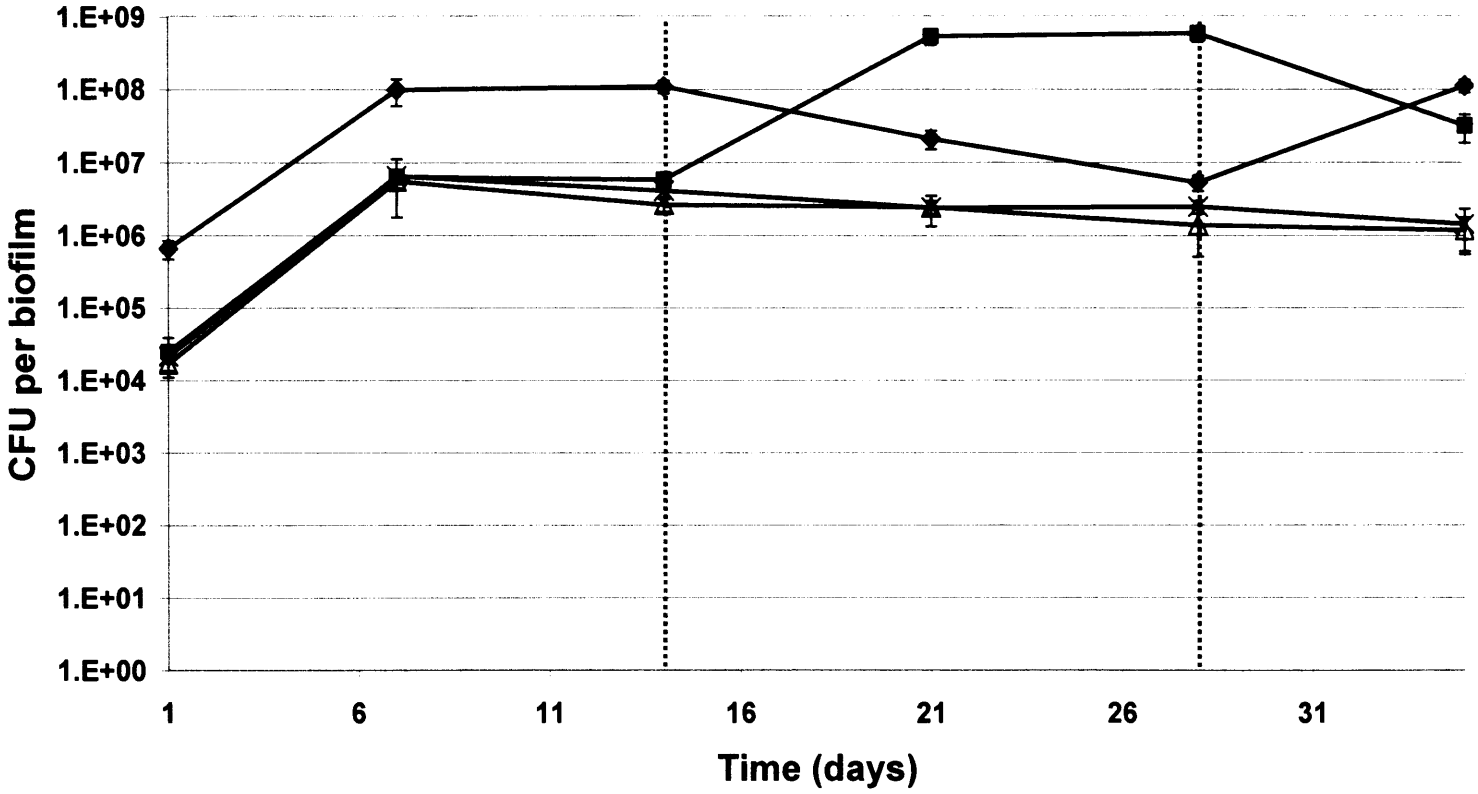

Figure 4.3.4: Viable counts from microcosm biofilms when gingivitis conditions were commenced on day 14 and then ceased on day 28.

■, *Actinomyces* spp.; ♦, *Streptococcus* spp.; Δ, *Veillonella* spp.; **x**, *Lactobacillus* spp. Error bars represent the standard deviations (n = 5).

Table 4.3.2 shows the selected species proportions from a typical microcosm dental plaque experiment before and after the addition of artificial GCF and microaerophilic gas. Before the change in environmental conditions, *Streptococcus* spp. dominated the biofilms and accounted for almost 60% of the total cultivable organisms present on the anaerobic agar plates. Following the change in conditions, *Actinomyces* was the most dominant genus, increasing from 4 to 70% of the total anaerobes. *Veillonella* and *Lactobacillus* proportions increased as did Gram-negative species, which showed a 10-fold increase and accounted for a greater proportion of cultivable species than *Streptococcus* spp.

	Biofilm formation (%)*	
Bacteria	**Health**	**Gingivitis**
***Streptococcus* spp.**	**58.4** (7.1)	**5.3** (0.9)
***Actinomyces* spp.**	**4.5** (0.3)	**70.1** (15.8)
***Lactobacillus* spp.**	**0.9** (0.2)	**2.1** (0.29)
***Veillonella* spp.**	**0.8** (0.1)	**1.5** (0.53)
Gram-negative species	**0.5** (0.04)	**5.7** (0.60)

Table 4.3.2: Proportions of selected bacterial species before and after induction of gingivitis conditions. * represents the percentage of total cultivable anaerobes at day 7 (before addition) and day 14 (after addition). Data are means and standard deviations (n = 4).

4.3.2 16S rRNA gene sequencing of cultured isolates

4.3.2.1 Non-selective media

All species identified under aerobic or anaerobic growth conditions on non-selective culture media during the course of several CDFF experiments are listed in Table 4.3.3. More species were identified on agar incubated anaerobically (27 species identified) than on agar incubated aerobically (12 species identified). However, most species which were isolated aerobically were also isolated under anaerobic conditions apart from *Citrobacter youngae* and *Brevibacillus agri*.

4.3.2.2 Selective media

Species of the desired genera were detected on selective media (Table 4.3.4). The greatest richness of species were isolated from agar selective for *Streptococcus* spp.

	Species identified
***Actinomyces* spp.**	*A. naeslundii, A. odontolyticus, A. viscosus*
***Streptococcus* spp.**	*S. mitis, S. salivarius, S. sanguinis, S. parasanguinis, S. pneumoniae*
***Lactobacillus* spp.**	*L. fermentum, L. fermentum/cellobiosus*
***Veillonella* spp.**	*V. dispar, V. parvula*
***Gram-negative* species**	*Pr. veroralis, V. parvula*

Table 4.3.3: Species identified on selective media. *Actinomyces* spp. were identified on CFAT agar; *Streptococcus* spp. on MS agar; *Lactobacillus* spp. on Rogosa agar; *Veillonella* spp. on Veillonella agar and Gram-negative species on CBA with a Gram-negative supplement.

4.3.2.3 Changes in oral microbiota associated with changes in environmental conditions

Biofilms were grown from a salivary inoculum and removed under conditions emulating health (at least 7 days old) and gingivitis. Additionally, samples were taken from the salivary inoculum. In total, 36 different taxa were identified (Table 4.3.5). The highest richness was detected in biofilms removed after gingivitis conditions were induced. However, a total of 16 taxa were detected uniquely in only one of the sample types (3 from saliva, 2 from health and 12 from gingivitis).

From the samples taken under conditions emulating health 16 taxa were identified. Of these 7 taxa were streptococci. *Gemella sanguinis* and *Streptococcus parasanguinis* were only isolated under these conditions. In contrast, after conditions were changed to emulate gingivitis, 29 taxa were identified, belonging to a wide range of genera with streptococci accounting for 8 taxa, a lower proportion of the total species detected than under conditions emulating health. Taxa not detected previously included; *Streptococcus anginosus, Streptococcus suis*, *Gemella sanguinis*, *Brevibacillus agri*, *A. defectiva, Veillonella dispar* and *Citrobacter* spp.

Only one taxa detected under conditions emulating health were *Actinomyces* spp. but under conditions emulating gingivitis 6 different taxa were detected. *A. odontolyticus* was the only *Actinomyces* spp. identified under conditions emulating health. In contrast, several *Actinomyces* spp. were detected under conditions emulating gingivitis. *A. naeslundii* and *A. viscosus* which were isolated from saliva samples were again isolated from samples taken after the induction of gingivitis conditions and *A. suimastitidis* and *A. turicensis* were only isolated after the induction of gingivitis conditions.

Species	Saliva	Health	Gingivitis
Firmicutes			
Brevibacillus agri [b]			±
Staphylococcus epidermidis/caprae	±		
Abiotrophia defectiva			±
Abiotrophia para-adiacens		+	+
Enterococcus faecalis [a]		+	+
Enterococcus sp. 8 [a]		+	+
Gemella haemolysans [a]		±	
Gemella sanguinis			±
Granulicatella adiacens		+	+
Lactobacillus casei/paracasei	±		
Lactobacillus fermentum/cellobiosus			±
Lactobacillus fermentum		+	+
Streptococcus anginosus [a]			±
Streptococcus genomosp. C8		+	+
Streptococcus gordonii/mitis	+		+
Streptococcus mitis group [a]	+	+	
Streptococcus mitis		+	+
Streptococcus oralis/mitis [a]		+	+
Streptococcus parasanguinis [a]		±	
Streptococcus pneumoniae			±
Streptococcus salivarius	+	+	
Streptococcus sanguinis [a]	+	+	+
Streptococcus suis			±
Veillonella dispar			±
Veillonella parvula	+	+	+
Actinobacteria			
Actinomyces graevenitzii	±		
Actinomyces lingnae	+		+
Actinomyces naeslundii	+		+
Actinomyces odontolyticus [a]		+	+

Actinomyces viscosus	+		+
Actinomyces suimastitidis			±
Actinomyces turicensis			±
Rothia dentocariosa [a]	+	+	+
Proteobacteria			
Citrobacter sp. 'genomospecies 11'			±
Citrobacter youngae [b]			±
Bacteroidetes			
Prevotella veroralis	+		+
TOTAL NUMBER OF ISOLATES	**13**	**16**	**29**
UNIQUE ISOLATES	**3**	**2**	**12**

Table 4.3.4: Cultivable taxa identified by 16S rRNA sequencing from saliva and plaque samples grown under conditions emulating health and gingivitis. ± represent species unique to that sampling point. All isolates were recovered under anaerobic conditions apart from [a], which were isolated both aerobically and anaerobically and [b] which were isolated aerobically.

4.3.3 Analysis of microcosm communities using CLSM

During the course of biofilm development different structural features could be observed (Fig. 4.3.5). Structures where large clusters of non-viable bacteria were surrounded by layers of viable bacteria (Fig. 4.3.5a) were often observed. Smaller clusters of viable and non-viable bacteria were also observed (Fig 4.3.5b). In mature biofilms non-viable bacteria were more widespread throughout the biofilm, not confined to layers beneath viable bacteria (Fig. 4.3.5c). Filamentous structures were also more commonly observed in more mature biofilms (Fig. 4.3.5d).

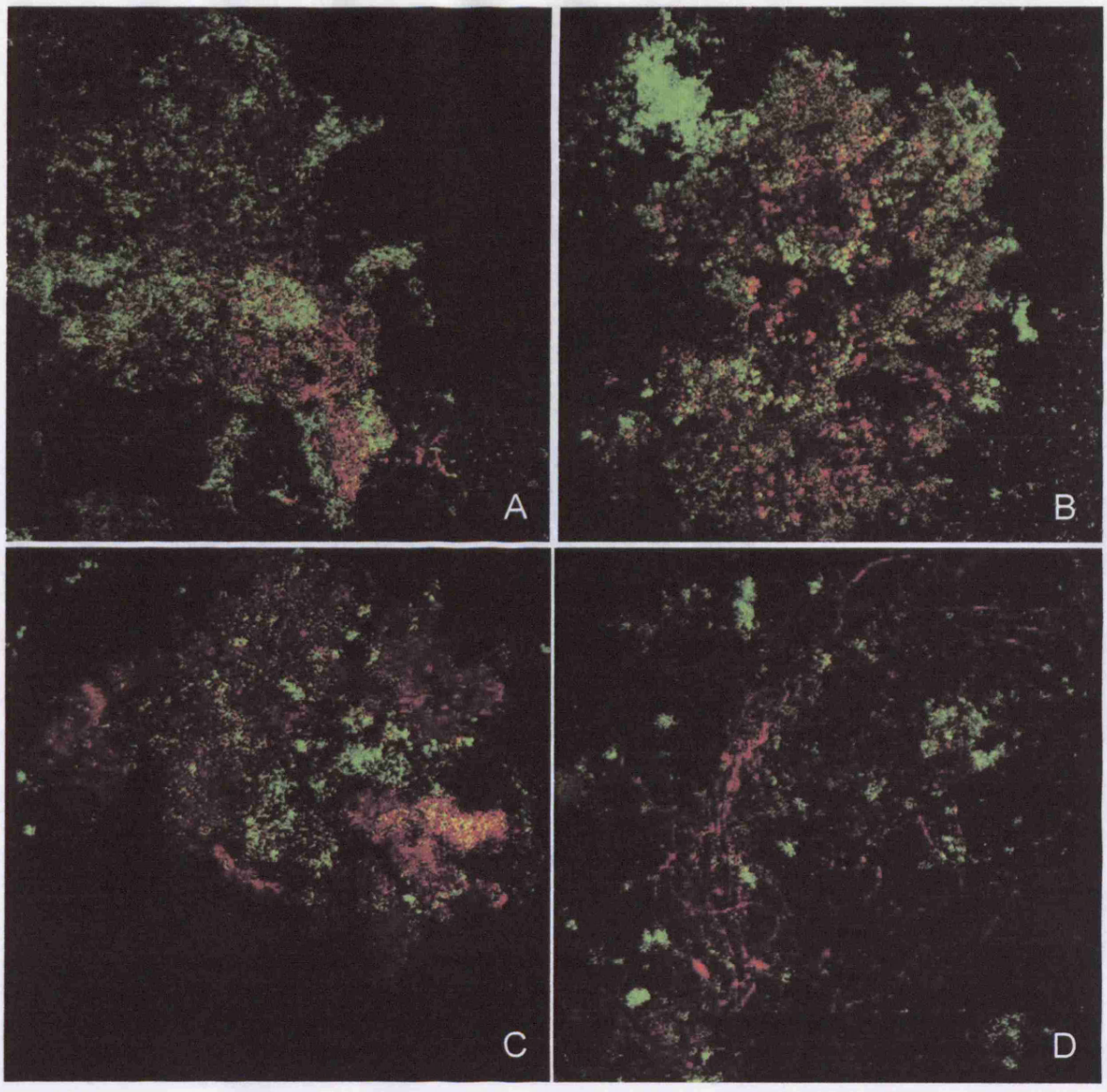

Figure 4.3.5: CLSM images along the X-Y plane (200 by 200 µm) of microcosm biofilms. A, taken from 5-day-old biofilm; B, taken from 7-day-old biofilm; C, taken from 14-day-old biofilm; D, taken from a 16-day-old biofilm. Images are a single *xy* section of the biofilm taken at depths of 15, 25, 21 and 44 µm respectively. Green represents viable bacteria, and red represents non-viable bacteria.

Under conditions emulating health, biofilms displayed an open structure with many visible voids and channels and structural features, such as filaments (Fig. 4.3.6). Non-viable bacteria presented a significant portion of the biofilm structure but were confined mainly to deeper layers closer to the hydroxyapatite surface.

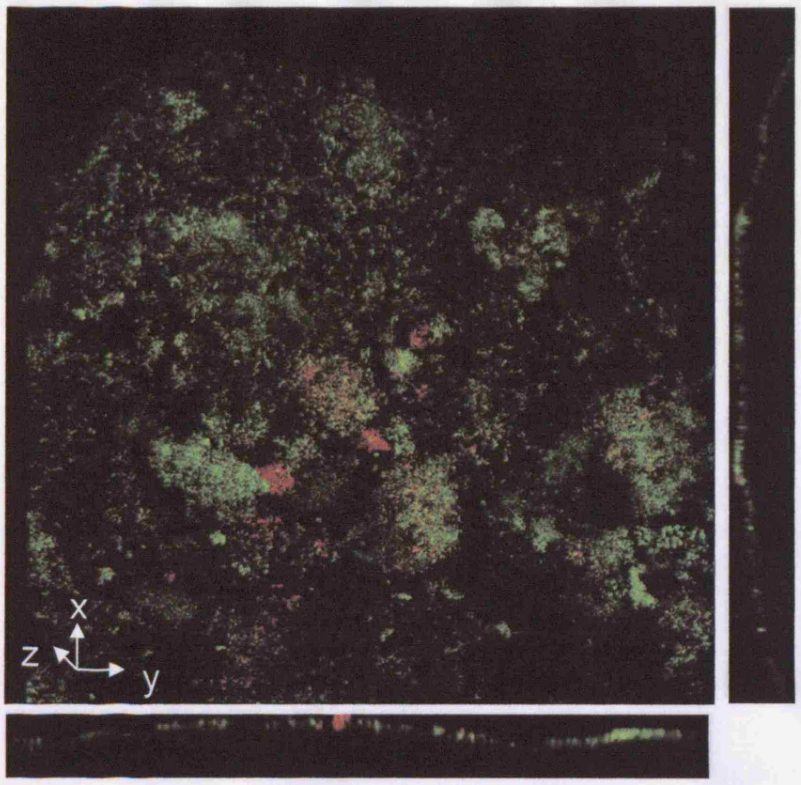

Figure 4.3.6: CLSM images (300 by 300 μm) of a 14-day-old microcosm biofilms grown under conditions emulating health. Green represents viable bacteria, and red represents nonviable bacteria. Images are *xy* sections taken through layers (1-3 μm) of the biofilm merged to create a 3D projection of the *z* axis. Arrows indicate directions of the *x*, *y* and *z* planes. Side panels are sagittal (*xz*) images of the biofilm taken from specific points along the *xy* axis.

After gingivitis conditions were commenced, biofilms displayed a more tightly packed structure with fewer visible voids (Figure 4.3.7). Non-viable bacteria appeared to form a more significant portion of the biofilm structure and were visible in the uppermost regions. Additionally, they appeared to form stacks composed of predominantly non-viable bacteria.

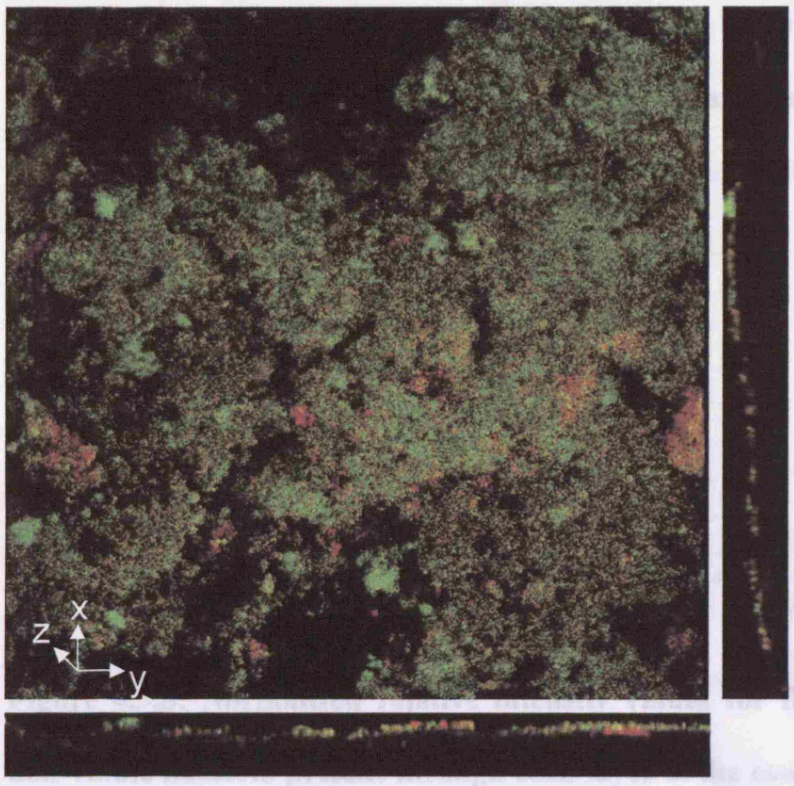

Figure 4.3.7: CLSM images (300 by 300 μm) of a 14-day-old microcosm biofilms grown under conditions emulating gingivitis. Green represents viable bacteria, and red represents nonviable bacteria. Images are *xy* sections taken through layers (1-3 μm) of the biofilm merged to create a 3D projection of the *z* axis. Arrows indicate directions of the *x*, *y* and *z* planes. Side panels are sagittal (*xz*) images of the biofilm taken from aspecific point along the *xy* axis.

Before the addition of artificial GCF and microaerophilic gas, non-viable bacteria demonstrated maximum fluorescence in the deeper layers of the biofilm, closer to the substratum, while the maximum fluorescence for viable bacteria was observed in the layers closer to the biofilm surface (Fig. 4.3.8). However, after the addition of artificial GCF and microaerophilic gas, both non-viable and viable bacteria showed maximum fluorescence in layers closer to the biofilm/air interface (Fig. 4.3.9). The relative

intensity values for viable and non-viable bacteria show a similar trend after the addition, with both showing reduced fluorescence toward the substratum surface.

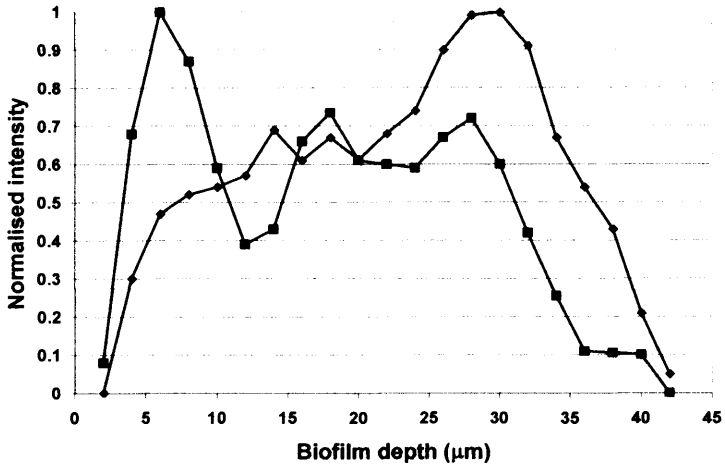

Figure 4.3.8: Normalized relative intensity values for fluorescence of viable and non-viable bacteria present through each layer of the confocal image of the biofilm under conditions emulating health. ■, viable bacteria; ♦, non-viable bacteria. Biofilm depth of 0 μm = air interface.

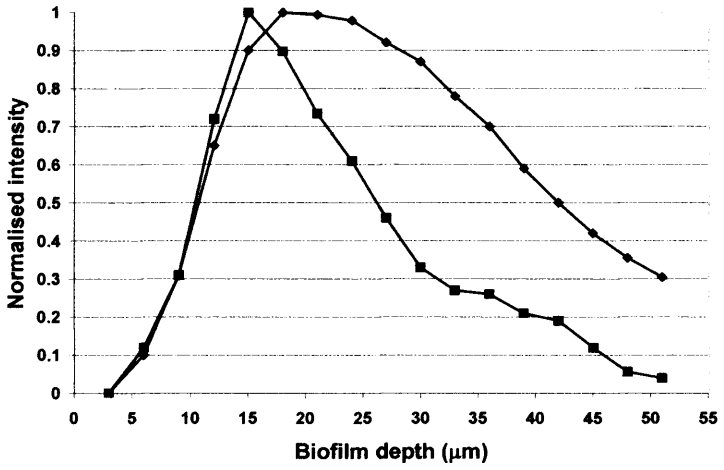

Figure 4.3.9: Normalized relative intensity values for fluorescence of viable and non-viable bacteria present through each layer of the confocal image **of the biofilm under conditions emulating gingivitis.** ■, viable bacteria; ♦, nonviable bacteria. Biofilm depth of 0 μm = air interface.

4.4 Discussion

Many bacteria which are implicated in disease are associated with the normal microbiota of humans. When these organisms spread from their natural site of colonization, are allowed to proliferate more rapidly, or exist in increased proportions due to changes in their environment, disease can result. One such example is dental plaque, the bacterial composition of which remains relatively stable. However, the ecological plaque hypothesis (Marsh 1994) proposes that this stability can be disrupted by major fluctuations in environmental factors, such as changes in diet, oral hygiene, and challenges by exogenous microbes, all of which are able to cause disease. Modeling changes in microbial compositions of dental plaque has previously focused on perturbation (McBain *et al,* 2003; Pratten *et al,* 1998a;b; Ready *et al,* 2002). The results of this work have shown that population shifts associated with gingivitis can be successfully modeled *in vitro* by mimicking changes in the oral environment.

4.4.1 Trends observed in species proportions

In the previous chapter, in a dual-species model, it was possible to demonstrate that by emulating the environmental conditions associated with gingivitis, it was possible to disrupt stable communities dominated by *S. sobrinus* to favour the growth of *A. naeslundii.* In this chapter when these changes were applied to the more complex microcosm model, the same shifts in *Streptococcus* and *Actinomyces* spp. proportions were observed, further establishing the link between these two environmental parameters and changes in microbial composition. In previous studies where the same *in vitro* model has been used to develop microcosm plaque with artificial saliva as the sole nutrient source, *Actinomyces* proportions ranged between 2 and 11% of the total cultivable flora (Pratten *et al,* 1998b; Pratten *et al,* 2003; Ready *et al,* 2002). With the

addition of artificial GCF and microaerophilic gas used in this study, *Actinomyces* spp. represented a much greater proportion (70%) of the total cultivable species. As this shift from a *Streptococcus*-dominated to an *Actinomyces*-dominated plaque is a common trend observed from *in vivo* experimental gingivitis studies (Moore & Moore, 1994; Syed & Loesche, 1978; Zee *et al,* 1996), it is significant that this relationship can be emulated using an *in vitro* model.

Although this chapter has focused on the relationship between *Streptococcus* and *Actinomyces* spp., Gram-negative species also play a significant role in biofilms grown under gingivitis conditions. This was shown by a 10-fold increase of these species as a proportion of the total cultivable organisms. The growth of Gram-negative anaerobes, such as *Tannerella* spp. (formerly *Bacteroides* spp.), *Prevotella* spp., and *F. nucleatum*, is enhanced by serum (ter Steeg *et al,* 1987), which is a major component of GCF. The addition of artificial GCF to the growth medium in these *in vitro* studies represented the introduction of a protein-rich nutrient source. The proteinase activity of some *Actinomyces* spp. is thought to play a role in the degradation of serum proteins into polypeptides for use by bacteria, such as *F. nucleatum* and *P. gingivalis* (Jansen & Van der Hoeven, 1997). The nutrients available in artificial GCF are not likely to be utilized directly by oral streptococci, such as *S. sanguinis* and *S. mitis*, which are carbohydrate dependent. *Actinomyces* spp. may also be more adaptive to changes in environmental conditions, due to their ability to utilize different metabolic pathways at a wider pH range than oral streptococci (Takahashi & Yamada, 1999). In general, increased nutrient availability will result in the formation of denser biofilms, most likely due to the increased production of extracellular polysaccharides by oral bacteria (Sutherland 2001),

which provides a further source of nutrients and plays a key role in biofilm architecture by providing a means for attachment for new species.

4.4.2 Characterisation of microbial communities before and after induction of gingivitis conditions

The aim of this study was to determine the richness of species present in the gingivitis CDFF model. The majority of bacteria isolated by culture were Gram-positive, facultative organisms. This is unsurprising as organisms which can grow under a wide range of conditions are likely to out-compete organisms which have more complex growth requirements when grown on non-selective media. The greatest number of species were identified from agar plates incubated under anaerobic conditions. These included strict anaerobes such as *Pr. veroralis* and *Veillonella* spp., species which prefer an anaerobic environment such as *Lactobacillus* spp. and *A. naeslundii* and facultative organisms such as *Abiotrophia* and *Granulicatella* spp.

There was an increase in species richness under conditions emulating gingivitis which involved the emergence of species not specifically associated with periodontal diseases. These included *Citrobacter* spp., which were only detected in samples taken after the induction of gingivitis conditions. These bacteria are not specifically associated with periodontal disease but have been isolated from other models of supragingival plaque (McBain *et al*, 2003). This increased diversity of species is indicative of the plaque environment itself becoming more varied.

16S rDNA sequencing of cultured isolates revealed changes in the dominant cultivable species in the microcosm plaque communities before and after induction of gingivitis

conditions. Before gingivitis conditions were introduced biofilms were dominated by a greater range of oral streptococci such as *S. mitis*, *S. sanguinis* and *S. salivarius*, species associated with gingival health while after addition *S. anginosus* was the most commonly isolated *Streptococcus* species. *S. anginosus* is more frequently detected in gingivitis samples than healthy plaque (Moore *et al,* 1982) showing that while the contribution of *Streptococcus* spp. to the microbiota as a whole is decreased as gingivitis develops not all species follow this trend.

A greater richness of *Actinomyces* spp. was identified by 16S rRNA gene sequencing. *A. naeslundii* and *A. viscosus* were both detected in saliva and also under conditions emulating gingivitis but not under conditions emulating gingival health. These organisms have previously been shown to be positively correlated with gingivitis (Moore *et al,* 1982; Moore & Moore, 1994; Syed & Loesche, 1978; Zee *et al,* 1996). *A. suismastidis* and *A. turicensis* were only detected in samples under gingivitis conditions. These organisms are not typically associated with gingivitis but again reflect the increased richness in microbial populations associated with the disease.

Species observed after gingivitis conditions were implemented included *R. dentocariosa* which has been linked to gingival recession (Tanner *et al,* 1998), the subgingival plaque associated with chronic periodontitis (Kumar *et al,* 2003, Colombo *et al,* 2002) and caries (Munson *et al,* 2004), *Citrobacter* spp. (Gram-negative facultatively anaerobic bacilli) which have been isolated in other microcosm models of dental plaque (McBain *et al,* 2003,2003b) and the subgingival plaque of elderly hospital patients (Preston *et al,* 1999) and *Abiotrophia* spp. which form part of the normal oral flora and are commonly wrongly identified as *Streptococcus* strains (Mikkelsen *et al,* 2000), even by 16S rDNA

sequencing. While these species are not linked to gingivitis they do reflect a change in the dominant cultivable species of microcosm plaques when exposed to new environmental factors. Gram-negative species more commonly associated with gingivitis such as *Prevotella* spp., *F. nucleatum* and *E. corrodens* were not detected.

4.4.3 Characterisation of biofilm architecture

Recent models for biofilm architecture propose a heterogeneous structure (Wimpenny *et al*, 2000; van Loosdrecht *et al*, 2002) which was observed in this study. Confocal microscopy has been applied to oral biofilms developed both *in situ* (Arweiler *et al,* 2004) and using *in vitro* models (Guggenheim *et al,* 2001b) to generate viability profiles. A characteristic feature of supragingival plaque is viable bacteria being less prevalent in deeper layers closer to the tooth surface (Arweiler *et al,* 2004; Auschill *et al,* 2001), and this distribution has also been observed from *in vitro* models (Hope & Wilson, 2003). This type of distribution was observed with biofilms in the present study before the change of environmental conditions to mimic gingivitis and suggests that non-viable bacteria may be a key feature both structurally and nutritionally for subsequent attachment of species. A common observation from confocal microscopy studies of dental biofilms is the heterogeneous nature of the biofilm structure (Guggenheim *et al,* 2001; Wood *et al,* 2000). Biofilm height and surface coverage are not uniform, and so bacteria, even at the deepest layers, can still be in contact with nutrients and oxygen. This type of structure may be more conducive to a plaque composition associated with oral health.

Under conditions emulating gingivitis, non-viable bacteria became more widely distributed throughout the plaque structure, showing a more significant presence in layers closer to the biofilm surface. This may reflect the development of

microenvironments within the biofilm structure (Hope *et al,* 2002) in which extreme gradients can exist across a small area. Vroom *et al,* (1999) demonstrated the presence of extreme niches in pH which developed as a response to the addition of sucrose. The introduction of artificial GCF and a microaerophilic environment may also instigate gradients in key environmental factors allowing the proliferation of species which may have had only a minor presence in biofilms grown under nutritionally limited conditions.

Due to the shear forces present within the model, biofilm height cannot exceed a specific depth. Thus, any bacterial proliferation occurring as a result of increased nutrient availability will have to occur within the limited space and may lead to the emergence of a more tightly packed structure. Previous studies (Pratten *et al,* 2000) have shown that the addition of sucrose to biofilms within this model can significantly change the coverage of microcosm plaque biofilms on hydroxyapatite, producing denser biofilms with fewer visible voids and channels and displaying a more uniform structure, which was suggested to be significant in the development of dental caries.

The increased presence of *Actinomyces* spp. in densely packed biofilms may be due to the specific structures formed. In a supragingival plaque model comprising of five-species (Guggenheim *et al,* 2001), *A. naeslundii* was shown to aggregate with most other species present and appeared to form structures spanning the height of the biofilm, coming into contact with the biofilm/air interface. *Streptococcus* spp. were shown to form horizontal structures and large microcolonies in the central strata of the biofilm. Single cells and chains were also present in mature biofilms. These differences in distribution may be critical to the survival of species as the biofilm structure changes. As biofilms become more-densely packed, *Actinomyces* spp. may have an advantage

spanning the height of the biofilm, allowing continued access to nutrients from the biofilm surface. The large microcolonies formed by *Streptococcus* spp. may be cut off from nutrient supplies and out-competed by organisms better able to grow with serum-derived nutrients.

4.4.4 Conclusions

In vitro models of microbial communities associated with health and disease are valuable tools for observing key factors in disease progression. When disease results from changes in the resident microbiota, the use of such models allows the influence of individual environmental factors to be assessed. While the richness of species present in this model could be assessed using the methods described in this chapter, quantitative information on individual species could not and the development of techniques to assess this will be described in the next chapter.

CHAPTER 5

Characterisation of microcosm communities using Quantitative PCR

5.1 Introduction

Historically, characterisation of the microbial populations associated with health and periodontal diseases in dental plaque has been carried out using exhaustive culture techniques involving the use of selective media and biochemical tests in order to enumerate and identify bacteria to the species level. However, many bacteria that are specifically associated with gingivitis are difficult to culture using these traditional techniques and therefore their numbers are often underestimated as members of the oral microbiota (Martin *et al,* 2002; Nonnenmacher *et al,* 2004; van Winkelhoff *et al,* 2002). These species may have complex nutritional requirements, slow growth rates or do not grow well on culture media in the presence of less fastidious organisms, in which case species or genera specific PCR can be more effective for detecting these organisms in a sample. As the development of periodontal diseases is linked to changes in proportions of several species, rather than particular species emerging after the development of a disease, it is more worthwhile to monitor changes in species numbers rather than simply detected their presence or absence in a sample. Quantitative PCR (qPCR) has the potential to do this.

As mentioned in Chapter 1 (Section 1.2) qPCR primers have been developed for several oral species to assess bacterial numbers in plaque samples. Some of these are summarized in Table 5.1.1. The majority of primers developed have targeted the 16S rRNA gene as this gene contains both highly conserved regions which are useful for the design of universal primers (Lane, 1991; Marchesi *et al,* 1998) and hypervariable regions which are ideal for the design of PCR primers for the unique identification of bacterial species (Tran and Rudney, 1996).

Species	Target	Reference
***Actinomyces* spp.**		
A. naeslundii	Unknown	Suzuki *et al,* 2004a
A. viscosus	Unknown	Suzuki *et al,* 2004a
***Streptococcus* spp. (universal)**	16S rRNA gene	Rudney *et al,* 2003
Streptococcus sobrinus	gtfB	Yoshida *et al,* 2003
Streptococcus mutans	Unknown	Yoshida *et al,* 2003
Streptococcus gordonii	Unknown	Suzuki *et al,* 2004a
Streptococcus mitis	16S rRNA gene	Suzuki *et al,* 2004a
***Prevotella* spp. (universal)**	16S rRNA gene	Martin *et al,* 2002
Pr. intermedia	16S rRNA gene	Maeda *et al,* 2003; Kuboniwa *et al,* 2004
	phoC (phosphotyrosyl phosphatase) gene	Ansai *et al,* 1998
Pr. melaninogenica	phyA (hemolysin) gene	Allison & Hillman, 1997
Pr. nigrescens	16S rRNA gene	Kuboniwa *et al,* 2004; Nagashima *et al,* 2005
P. gingivalis	16S rRNA gene	Kuboniwa *et al,* 2004; Maeda *et al,* 2003; Martin *et al,* 2002
	Arg-gingipain	Morillo *et al,* 2003
	FimA (fimbrial) gene	Doungudomdacha *et al,* 2000
A. actinomycetemcomitans	16S rRNA gene	Kuboniwa *et al,* 2004; Boutaga *et al,* 2005
	lktA (leukotoxin A) gene	Doungudomdacha *et al,* 2000
	lktC (leukotoxin C) gene	Morillo *et al,* 2003
***Fusobacterium* spp.**	16S rRNA gene	Martin *et al,* 2002; Suzuki *et al,* 2004b

T. forsythia	16S rRNA gene	Kuboniwa *et al,* 2004; Suzuki *et al,* 2004b; Boutaga *et al,* 2005
T. denticola	16S rRNA gene	Kuboniwa *et al,* 2004

Table 5.1.1: qPCR primers developed for oral bacteria.

From the previous chapters *Actinomyces* and *Streptococcus* spp. have been shown to be important species in the oral biofilm communities developed in the CDFF associated with health and gingivitis. Gram-negative species however were less frequently detected and perhaps underestimated by the culture techniques used. This chapter describes attempts to develop qPCR primers to target species or genera thought to be of interest in oral biofilms associated with health or gingivitis. Individual *Actinomyces* and *Streptococcus* spp. were thought to be of particular interest as were Gram-negative species such as *Fusobacterium* and *Prevotella* spp. due to these organisms being rarely detected, if at all, by the culture techniques used in Chapter 4.

5.2 Materials and Methods

5.2.1 Evaluation of qPCR primers

5.2.1.1 Bacterial strains and growth conditions

DNA extracted from *A. naeslundii* NCTC 10301, *A. viscosus* NCTC 10951, *A. odontolyticus* NCTC 9935, *A. israelii* NCTC 10236, *S. sanguinis* NCTC 7863, *S. parasanguinis* NCTC 55898, *S. sobrinus* NCTC 12279, *S. gordonii* NCTC 7865, *S. oralis* NCTC 11427, *S. cristatus* CR311, *S. mutans* NCTC 10499, *S. salivarius* NCTC 8618, *S. anginosus* NCTC 10713, *S. mitis* NCTC 12261, and oral isolates of *F. nucleatum*, *P. gingivalis*, *Pr. intermedia*, *E. faecalis*, *G. haemolysans*, *C. youngae*, *A. defectiva* and *R. dentocariosa* were used as a panel of oral species to test the specificity of the qPCR primers. DNA was extracted from known numbers of bacteria. Bacterial numbers were obtained by enumerating the CFUs from cultures of each species grown in BHI broth (Oxoid) at 37°C under anaerobic conditions for 3 to 4 days.

5.2.1.2 Comparison of primer sequences to BLAST database

To ensure that potential primers were specific for their intended target their sequences were compared to the BLAST database.

5.2.1.3 Conventional PCR

The specificity of potential primer sets for their desired target was assessed using conventional PCR (Yoshida *et al,* 2003) against the panel of oral species mentioned above. The PCR master mix consisted of 34.3 µl of molecular grade H_2O (Sigma), 5.0 µl of 10X buffer (Bioline, London, UK), 1.5 µl of $MgCl_2$ (Bioline), 1.0 µl of 10 µM dNTP mixture (Bioline), 1.5 µl each of forward and reverse primers (10 µM, Sigma-Genosys), 0.2 µl of BioTaq (BIOLINE) and 5.0 µl of DNA. This was then run on the

following PCR program, an initial step of 94°C for 5min, followed by 25 cycles of 94°C for 15 s, 55°C 30 s and 72°C for 1 min (Biometra T3000, Goettingen, Germany). The products were then run on a 3% agarose gel and products examined by UV illumination to confirm that only one product of the appropriate length was produced.

5.2.1.4 qPCR Assay

To test the specificity of qPCR assays were carried out as described in Chapter 2 (Section 2.5) against the target species and the panel of oral species mentioned above.

5.2.2 qPCR Primers for oral bacteria

5.2.2.1 Existing qPCR primers

As qPCR primers had already been designed for several oral species the first approach was to assess these existing primers for species that were thought to be of interest in this model. All the primers sets listed in Table 5.2.1 were tested against their target species and against a panel of negative controls by conventional PCR.

Species	Reference	Outcome
A. naeslundii	Suzuki *et al,* 2004a	No product
A. viscosus	Suzuki *et al,* 2004a	No product
S. sobrinus	Yoshida *et al,* 2003	No product
S. gordonii	Yoshida *et al,* 2003	Cross reactions with other species.
S. mitis	Yoshida *et al,* 2003	Cross reactions with other species.
***Streptococcus* spp.**	Rudney *et al,* 2003	Suitable for use
***Prevotella* spp.**	Martin *et al,* 2002	Suitable for use
***Fusobacterium* spp.**	Martin *et al,* 2002	Suitable for use
P. gingivalis	Maeda *et al,* 2003	Suitable for use
***Treponema* spp.**	Asai *et al,* 2002	Non-specific products.
Universal	Martin *et al,* 2002	Suitable for use

Table 5.2.1: qPCR primers tested for specificity for their target species.

5.2.2.2 Design of qPCR primers

As the previously designed qPCR primers for *Actinomyces* spp. (Suzuki *et al,* 2004a), along with those for several other species, did not produce a specific product new qPCR primers were designed. For primers to work efficiently in a qPCR assay it was necessary for them to adhere to a strict set of parameters (Table 5.2.2).

Parameter	Limits
Amplicon size	50 – 150 bp
G + C content	Close to 50%
Melting temperature T*m*	Between 58 -60°C
Difference between primer T*m*	Less than 5°C
Gs and Cs in last 5 bases	No more than three
Runs of more than 3 of the same base	Avoid
Specificity	Specific for intended species/ genera
Secondary structure formation	Avoid

Table 5.2.2: Parameters for the design of qPCR primers.

In order to find a specific region as a candidate for species or genera specific primers the desired sequence was downloaded from BLAST, along with sequences of closely related species or genera. These sequences were then aligned and compared using the Clustal W program to highlight regions of specificity. Candidate regions of interest were loaded into primer designing software (www.idtdna.com/Scitools/Applications/Primerquest/) to generate potential primer pairs. Candidate primers were then compared to the BLAST database to ensure their specificity for their intended target.

5.2.2.2.1 Primers based on the 16S rRNA gene

The 16S rRNA gene for *Actinomyces* spp. was compared using the Clustal W program for *A. naeslundii* (Accession no. M33911.1), *A. viscosus* (AF543286.1), *A. odontolyticus* (AJ234040.1) and *A. israelii* (X82450.1), revealing species-specific

regions. Primers were designed based on these regions and the parameters required for qPCR primers whilst still being specific for their target species. Figure 5.2.1 demonstrates the species-specific regions used as targets for *A. naeslundii* specific primers. Both primers were found to be specific for *A. naeslundii* by searching the BLAST database.

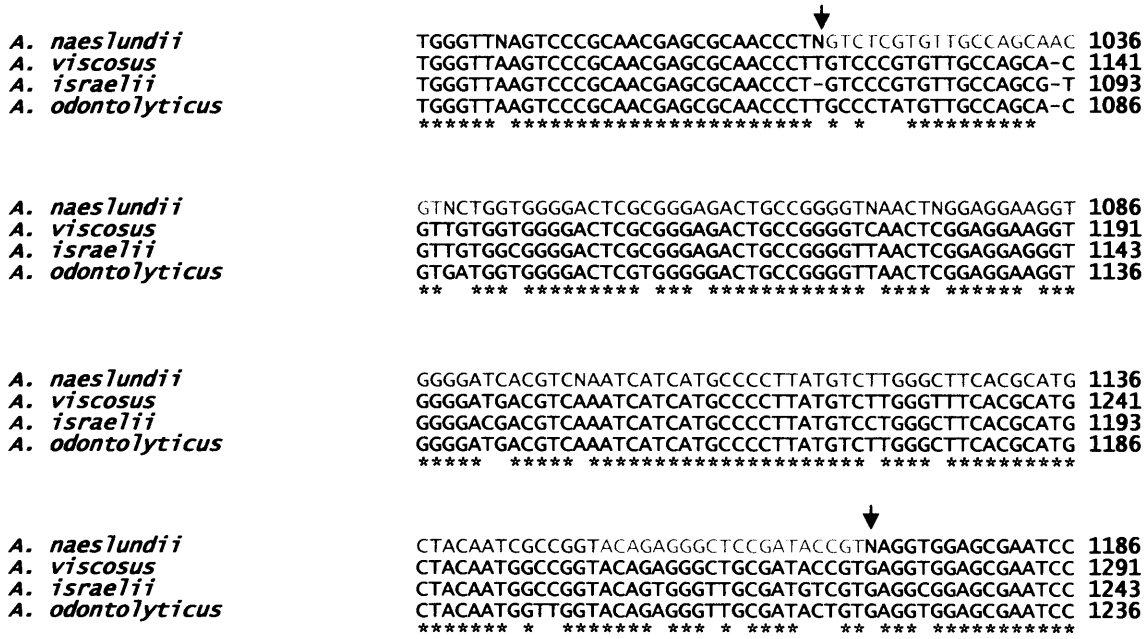

Figure 5.2.1: Regions used as targets for *A. naeslundii* specific primers based on alignment of the 16S rRNA gene for *Actinomyces* spp. (ATCG represent target sequences for primer design; ATCG represent region amplified between primers; ** represent identical aligned sequences; -- represent gaps in aligned sequences).

Figure 5.2.2 demonstrates the species-specific regions used as targets for *A. viscosus* specific primers. Only the forward primer was shown to be specific for *A. viscosus* by searching the BLAST database.

Figure 5.2.2: Regions used as targets for *A. viscosus* specific primers based on alignment of the 16S rRNA gene for *Actinomyces* spp. (ATCG represent target sequences for primer design; ATCG represent region amplified between primers; ** represent identical aligned sequences; -- represent gaps in aligned sequences).

Figure 5.2.3 demonstrates the species-specific regions used as targets for *A. odontolyticus* specific primers. Only the forward primer was shown to be specific for *A. odontolyticus* by searching the BLAST database.

Figure 5.2.3: Regions used as targets for *A. odontolyticus* specific primers based on alignment of the 16S rRNA gene for *Actinomyces* spp. (ATCG represent target sequences for primer design; ATCG represent region amplified between primers; ** represent identical aligned sequences; -- represent gaps in aligned sequences).

Figure 5.2.4 demonstrates the species-specific regions used as targets for *A. israelii* specific primers. Both primers were found to be specific for *A. israelii* by searching the BLAST database.

Figure 5.2.4: Regions used as targets for *A. israelii* specific primers based on alignment of the 16S rRNA gene for *Actinomyces* spp. (ATCG represent target sequences for primer design; ATCG represent region amplified between primers; ** represent identical aligned sequences; -- represent gaps in aligned sequences).

Species	Primer	Sequence (5' to 3')	Tm (°C)	Amplicon size (bp)
Actinomyces	Forward	GTCTCGTGTTTGCCAGCAACGT	60.6	152
naeslundii	Reverse	ACGGTATCGGAGCCCTCTGT	60.2	
Actinomyces	Forward	ATGCGCCTTTGGGTGTT	55.5	108
viscosus	Reverse	GTCCACCCTCTCAGGCCG	60.6	
Actinomyces	Forward	ATGCGCCTTTGGGTGTT	55.5	131
odontolyticus	Reverse	CATCACGTGCTGGCAACATAG	56.5	
Actinomyces	Forward	AAGTCGAACGGGTCTGCCTTGT	61.0	160
israelii	Reverse	GTGAATCTTTCCCAGCCACACCAT	59.6	

Table 5.2.3: qPCR Primers designed for *Actinomyces* spp.

Table 5.2.3 describes the species-specific primers designed for *Actinomyces* spp. The proposed primer sets were tested by conventional PCR to confirm that there was a product of the desired length and that there were no cross reaction with other *Actinomyces* spp. When qPCR was attempted with these primers analysis of the PCR products revealed the presence of non-specific products with other *Actinomyces* spp., the formation of which could not be prevented by altering the reagent or PCR cycling conditions. As these products would significantly affect the accuracy and interpretation of qPCR results these primers were thought to be unsuitable.

5.2.2.2.2 Primers based on the Intergenic Spacer (ITS) region

As attempts to develop primers based on the 16S rRNA gene were unsuccessful, due to the similarity of the 16S rRNA gene in this region between different species of the *Actinomyces* genera, other unique regions would have to be identified. The intergenic spacer region between the 16S and 23S rRNA gene was thought to be a good region to explore for primer design. This region shows a greater degree of variability between species than the 16S rRNA gene and thus would be more likely to have unique regions for primer design. As this region has not yet been sequenced for many bacteria the first step was to amplify and sequence this region for different *Actinomyces* spp. in order to find any unique regions for primer design. Three sets of universal primers (Table 5.2.2) were used to amplify this region in several oral species of interest.

Primer	Sequence	Reference
1406F	5'-TGYACACACCGCCCGT-3'	Cardinale
23SR	5'-GGGTTBCCCCATTCRG-3'	*et al,* 2004
ITSF	5'-GTCGTAACAAGGTAGCCGTA-3'	Cardinale
ITSReub	5'-GCCAAGGCATCCACC-3'	*et al,* 2004
785F	5'-GGATTAGATACCCTGGTAGTC-3'	Lyons *et*
422R	5'-GGAGTATTTAGCCTT-3'	*al,* 2000

Table 5.2.4: Universal primers for the ITS region.

This region could not be amplified for *A. naeslundii* and *A. israelii* using any of the universal primer sets tested. As ascertaining unique primers for *A. naeslundii* and *A. viscosus* was the aim with this approach being unable to amplify this region in *A. naeslundii* meant this approach had to be abandoned.

5.2.2.3 Modification of existing PCR primers for qPCR

Species or genera specific primers designed for endpoint PCR (Table 5.2.3) were modified and combined with alternative primers to better suit the parameters for qPCR primers and to produce an amplicon length suitable for qPCR. Table 5.2.3 summarises some of the primer sets available for modification.

Species	Sequence (5' to 3')	T*m* (°C)	Amplicon size (bp)
A. naeslundii	GCGCCTTTTTTGGTGTTTTTGG	56.5	274
	CACCCACAAACGAGGCAGGCCTG	64.9	
A. viscosus	GTGAAGGAGCCAGCTTGCTGGTTCGT G	65.1	155
	CGGAACAAACCTTTCCCAGGC	59.0	
A. israelii	GGTCTGCCTTGTTTTTTGCGGGGTGG G	66.0	439
	CATAACCCGGCTACCGTCAACC	60.0	
Capnocytophaga **spp.**	GGATAGCCCGAAGAAATTTGGAT	55.2	337
	CGTCATCAAAGTACACGTACTCCTTA T	52.3	
Streptococcus ***anginosus*** **group**	TGCTGCAACGGTAGCTAATGG	57.8	275
	CAAAGGTTTCTGCTGTCCCTG	56.3	

Table 5.2.5: Species-specific primers designed for *Actinomyces* spp. based on the 16SrRNA gene (Xia and Baumgartner 2003), for *Capnocytophaga* spp. (Fredricks *et al,* 2005) and the anginosus group of the genus *Streptococcus* (Takao *et al,* 2004) based on the penicillin-binding protein gene.

The primers listed in Table 5.2.3 were tested for their specificity for their target using standard PCR. Whilst specific, none of these primers adhered to the parameters required for qPCR or produced an amplicon of the optimum length and so alternative primer combinations were investigated. For the *A. naeslundii* primers the forward

primer was unsuitable due to a low G + C content and runs of more than 3 of the same base and so the reverse primer was combined with other potential primers for use in qPCR. In its original form this primer was not optimal for qPCR as the G + C content and melting temperature were too high, so this primer was shortened to give a lower G + C content and melting temperature closer to the optimum required for qPCR. This primer was used with the universal forward primer 357F (Lane, 1991) to give an amplicon within the optimum length range (134 bp). When tested by conventional PCR and qPCR this primer set was found to be specific for *A. naeslundii*. The *A. viscosus* primers in Table 5.2 produced an amplicon just out the optimum product length range and the difference between the melting temperatures of the primers was greater than 5 ^{o}C, with the forward primer also being out of the optimum length range. Attempts to modify these primers or combining them with alternative primers to make them suitable for a qPCR assay were unsuccessful. The amplicon produced by the *A. israelii* primers was too long and the forward primer unsuitable for use in a qPCR assay due to runs of more than 3 of the same base. Alternative forward primers were tried but all produced products with other *Actinomyces* spp.

The *Capnocytophaga* spp. specific primers were unsuitable for use in a qPCR assay due to their low T*m* and large product size. These primers were modified to fit the parameters for qPCR and combined with alternative forward and reverse primers but did not produce a specific product with any of the combinations tried.

The primers for the anginosus group of *Streptococcus* produced a large product and so alternative reverse primers were designed based on the penicillin-binding protein (pbp) gene. Whilst these primers were specific when assessed by conventional PCR and

qPCR, analysis of the dissociation curves revealed the production of non-specific products which would interfere with getting accurate results. Attempts to alter the qPCR assay to minimize this were also unsuccessful.

5.2.3 Analysis of biofilm samples by qPCR

5.2.3.1 Biofilm production

Biofilms were produced under conditions emulating health and gingivitis and stored as described in Chapter 2. Samples from replicate fermenters were pooled to get representative samples.

5.2.3.2 DNA extraction

Genomic DNA from bacteria, saliva and plaque samples was extracted as described in Chapter 2 (Section 2.4). For qPCR analysis plaque samples from fermenters grown under conditions emulating health and gingivitis were used.

5.2.3.3 qPCR assay

The primers which were found to be suitable for qPCR and used to analyse biofilm samples are listed in Table 5.2.6.

Species	Primer	Sequence (5' to 3')	Reference
***Prevotella* spp.**	Forward	CCAGCCAAGTAGCGTGCA	Martin *et al,* 2002
	Reverse	TGGACCTTCCGTATTACCGC	
***Fusobacterium* spp.**	Forward	AAGCGCGTCTAGGTGGTTAT GT	Martin *et al,* 2002
	Reverse	TGTAGTTCCGCTTACCTCTCC AG	
***Streptococcus* spp.**	Forward	AGATGGACCTGCGTTGT	Rudney *et al,* 2003
	Reverse	GCTGCCTCCCGTAGGAGTCT	
P. gingivalis	Forward	CTTGACTTCAGTGGCGGCAG	Maeda *et al,* 2003
	Reverse	AGGGAAGACGGTTTTCACCA	
A. naeslundii	Forward	CTCCTACGGGAGGCAGCAG	Lane, 1991
	Reverse	CACCCACAAACGAGGCAG	Xia & Baumgartner, 2003
Universal	Forward	TCCTACGGGAGGCAGCAGT	Martin *et al,* 2002
	Reverse	GGACTACCAGGGTATCTAAT CCTGTT	

Table 5.2.6: qPCR primers used to analyse plaque samples.

The qPCR plates were set up as described in Chapter 2 (Section 2.5) using primers at a concentration of 300 nM. The species used to generate standard curves for qPCR assays are listed in Table 5.2.7.

Primer Target	Species used for standard curve (Extracted DNA)
***Prevotella* spp.**	*Pr. intermedia*
***Fusobacterium* spp.**	*F. nucleatum*
Streptococcus spp.	*Streptococcus sanguinis* NCTC 7863, *S. parasanguinis* NCTC 55898, *S. sobrinus* NCTC 12279, *S. gordonii* NCTC 7865, *S. oralis* NCTC 11427, *S. cristatus* CR311, *S. mutans* NCTC 10499, *S. salivarius* NCTC 8618, *S. anginosus* NCTC 10713, *S. mitis* NCTC 12261
P. gingivalis	*P. gingivalis*
A. naeslundii	*A. naeslundii* NCTC 10301
Universal	*Actinomyces naeslundii* NCTC 10301, *Streptococcus sanguinis* NCTC 7863, *S. anginosus* NCTC 10713 and oral isolates of *F. nucleatum*, *P. gingivalis*, *Pr. intermedia*, *E. faecalis*, *G. haemolysans*, *C. youngae*, *A. defectiva*, *R. dentocariosa*

Table 5.2.7: Species used to create standard curves for qPCR assays.

For species or genera specific primers it was sufficient to use the DNA extracted from one species to generate standard curves. For universal primers it was necessary to pool the DNA from several oral species to generate standard curves as this primer was based on the 16S rRNA gene which has variable copy number in different species. To test the accuracy of detection of bacterial numbers using universal primers DNA extracted from a known number of bacterial cells for oral isolates of *F. nucleatum*, *P. gingivalis*, *Pr.*

intermedia, *E. faecalis*, *G. haemolysans*, *C. youngae*, *A. defectiva*, *R. dentocariosa* was tested in a qPCR assay using universal primers to ensure that the correct bacterial numbers were being detected.

5.3 Results

5.3.1 qPCR primers

5.3.1.1 Specificity of primers

The primer sets for *Streptococcus* spp. *Fusobacterium* spp., *Prevotella* spp., *P. gingivalis* and *A. naeslundii* were found to be specific for their desired species or genera without the production of primer dimers or non-specific products using qPCR assays.

5.3.1.2 Accuracy of universal primers

DNA extracted from known numbers of *F. nucleatum*, *P. gingivalis*, *Pr. intermedia*, *E. faecalis*, *G. haemolysans*, *C. youngae*, *A. defectiva* and *R. dentocariosa* were used to test the accuracy of universal primers. The universal primers detected the corresponding numbers of bacteria in both single and mixed species samples.

5.3.2 Saliva and Inoculum composition

All species or genera enumerated by qPCR were present in the pooled saliva inoculum and maintained at detectable, but reduced, levels during the 8 hour inoculation period (Fig. 5.3.1). In saliva, the most dominant genus detected was *Prevotella* spp. whilst in the 8 hour inoculum streptococci were dominant.

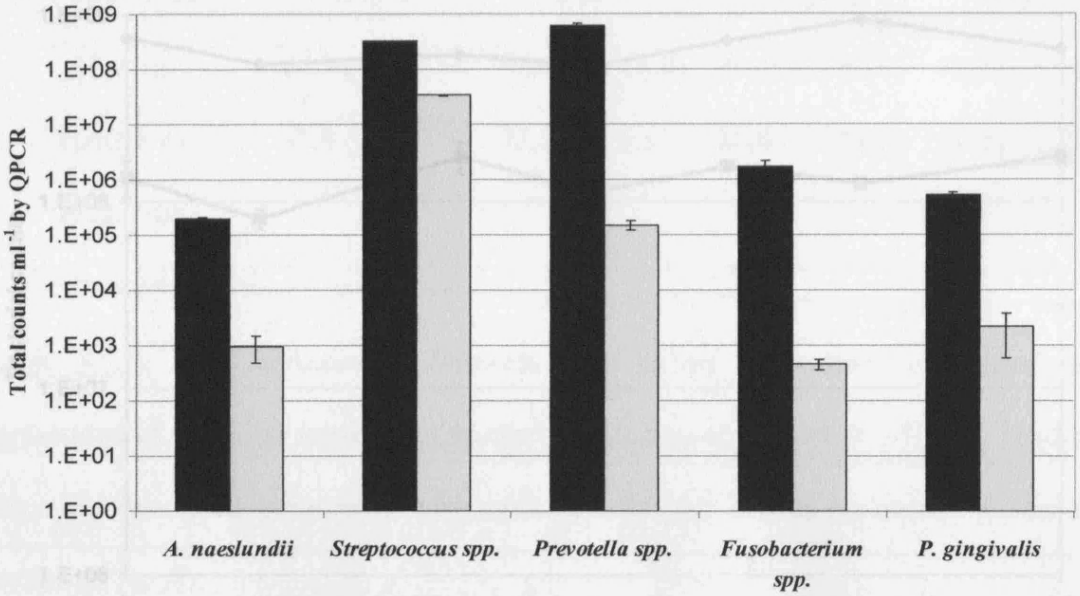

Figure 5.3.1: Total counts obtained by qPCR for pooled saliva and the 8 hour inoculum. ■, represents counts in saliva; ■, represents counts in 8 hour inoculum. Error bars represent standard deviations ($n = 4$).

5.3.3 Quantification of total bacteria by qPCR compared to culture

The total bacterial ribosomal RNA was quantified using universal primers for the 16S rRNA region. The corresponding estimates for the total bacteria present in the biofilm samples was then calculated by comparison to standard curves generated from serial dilutions of DNA extracted from known numbers of bacteria. The total number of bacteria determined by qPCR was always significantly higher ($P < 0.05$) than the total bacterial counts determined by culture (Fig. 5.3.2) regardless of the stage of biofilm development or the environmental conditions.

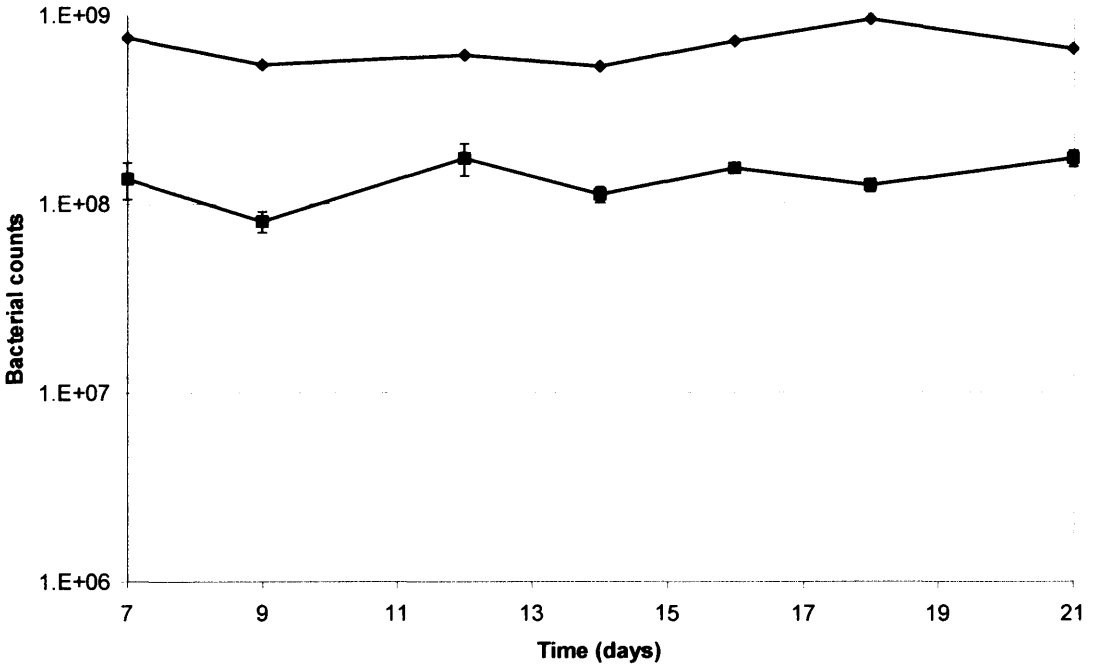

Figure 5.3.2: Total number of bacteria determined by culture and by qPCR when conditions emulating gingivitis were commenced on day 9. ■, CFU obtained by culture on FAA; ♦, total bacteria calculated by qPCR. Error bars represent standard deviations ($n = 4$).

The percentage of bacteria accounted for by culture compared to the total number of bacteria determined by qPCR decreased over time under conditions emulating health or gingivitis. There was no significant difference between the percentage of cultivable bacteria under conditions emulating health or gingivitis on any of the days sampled (Table 5.3.1).

Conditions	Day 1	Day 7	Day 14	Day 21
Health	**44.7** (+/- 11.0)	**31.9** (+/- 14.8)	**30.1** (+/- 6.10)	**25.0** (+/- 11.4)
Health to gingivitis	**41.0** (+/- 21.8)	**32.2** (+/- 6.51)	**28.0** (+/- 13.2)	**18.0** (+/- 12.5)

Table 5.3.1: Total number of bacteria determined by culture expressed as a percentage of the total number of bacteria determined by qPCR. Under 'Health to gingivitis' conditions parameters were switched after day 7. Data are means +/- standard deviations (n =8).

5.3.4 Species detected by qPCR

5.3.4.1 *Actinomyces* and *Streptococcus* spp. comparison with culture

Actinomyces spp. counts on selective media were slightly higher than numbers of *A. naeslundii* determined by qPCR (Fig. 5.3.3). Under conditions emulating health total numbers of *A. naeslundii* determined by qPCR ranged between 1.3 and 1.7 x 10^7 bacteria. Under conditions emulating gingivitis *A. naeslundii* numbers determined by qPCR ranged between 2.1 and 2.2 x 10^8 bacteria, representing a significant increase ($P < 0.001$).

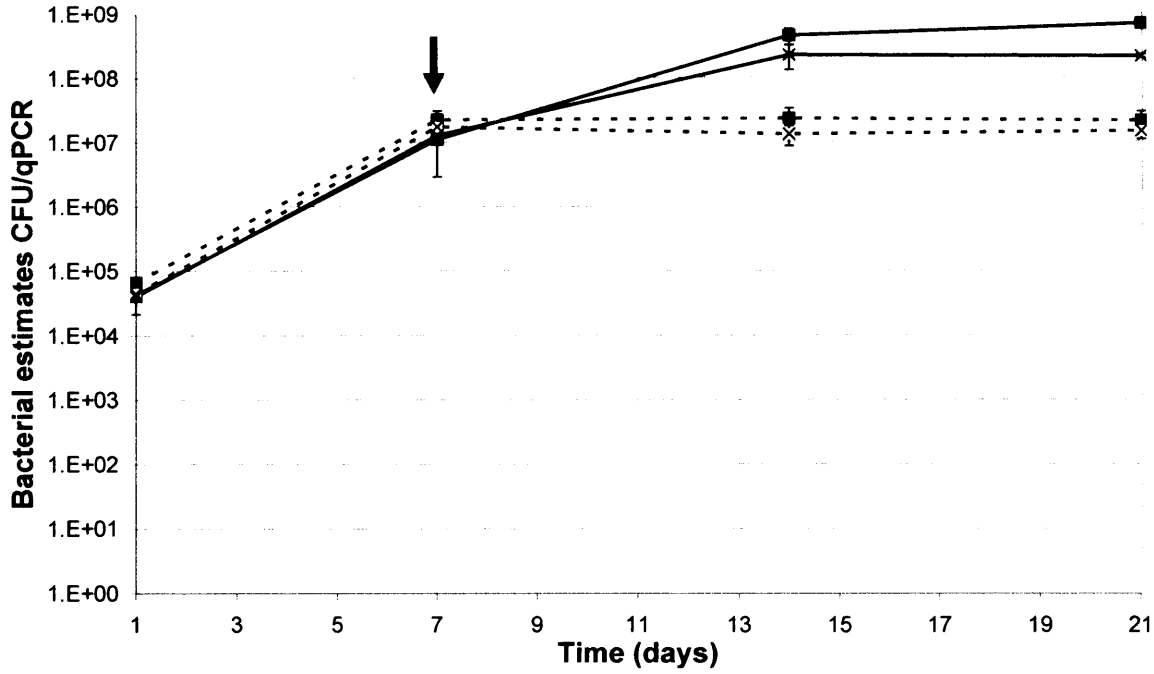

Figure 5.3.3: Comprison of total counts for *Actinomyces* spp. determined by culture and qPCR. ■ *Actinomyces* spp. CFU; × *A. naeslundii* qPCR estimates. Solid lines represent experiments where gingivitis conditions were commenced on day 7 (indicated by arrow). Dashed lines represent experiments where no change in conditions was implemented. Error bars represent standard deviations (n = 8).

Streptococcus spp. quantification as assessed by qPCR was consistently higher than that obtained on selective media for *Streptococcus* spp. (Fig. 5.3.4). Under conditions emulating gingivitis the numbers detected by both methods decreased significantly. Under conditions emulating health total counts for *Streptococcus* spp. determined by qPCR ranged between 2.2 and 4.3 x 10^8 bacteria. Under conditions emulating gingivitis the counts ranged between 6.1 and 7.8 x 10^7 bacteria representing a significant reduction ($P < 0.01$). The reduction in total counts for *Streptococcus* spp. when assessed by culture on selective media was more significant ($P < 0.001$), with numbers

decreasing from 2.3 (+/- 0.69) x 10^8 to 9.9 (+/- 6.0) x 10^6 bacteria, over a $\log_{10}$ reduction.

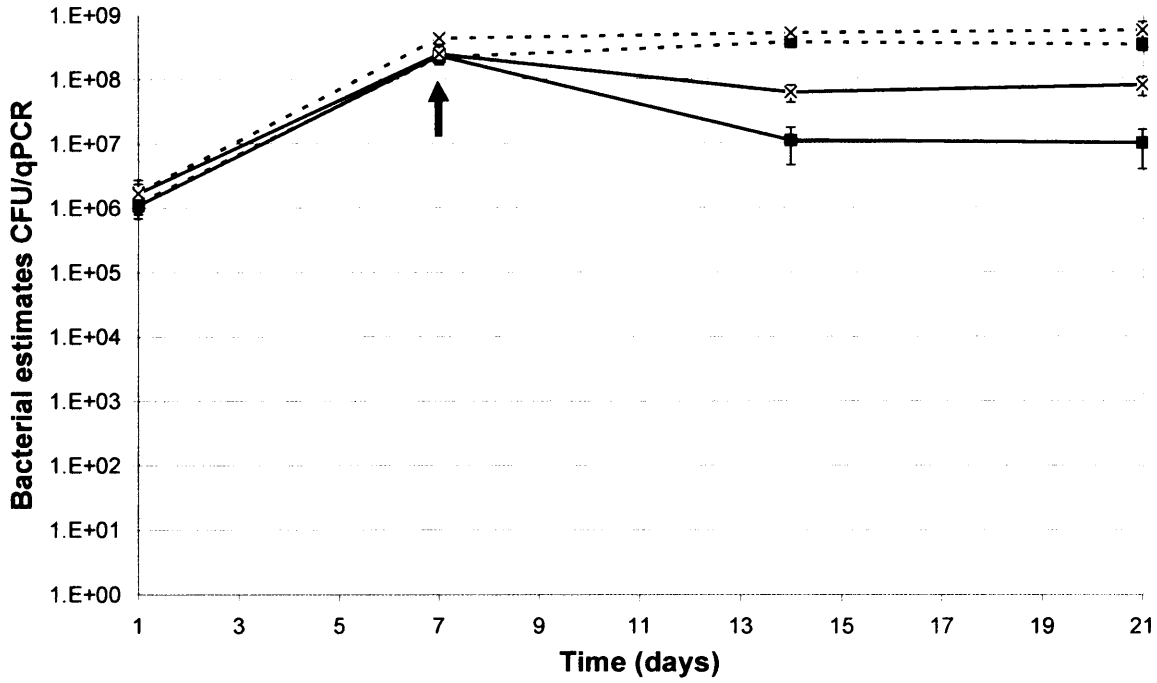

Figure 5.3.4: Comprison of total counts for *Streptococcus* spp. determined by culture and qPCR. ■ *Streptococcus* spp. CFU; × *Streptococcus* spp. qPCR estimates. Solid lines represent experiments where gingivitis conditions were commenced on day 7 (indicated by arrow). Dashed lines represent experiments where no change in conditions was implemented. Error bars represent standard deviations (n = 8).

5.3.4.2 Gram-negative species

Under conditions emulating health, on day 7, the total counts for *Prevotella* spp. determined by qPCR ranged between 3.1 and 3.6 x 10^7 bacteria (Fig. 5.3.5). When conditions were switched to emulate gingivitis after day 7 the total counts for *Prevotella* spp. increased reaching numbers of 9.9 (+/- 1.1) x 10^8 by day 21, representing a significant increase($P < 0.01$). In experiments where conditions emulating health were maintained throughout the total counts for *Prevotella* spp. determined by qPCR

continued to increase throughout the course of the experiment with a maximum number of 1.1 (+/- 0.1) x 10^8 being detected on day 21 of biofilm development. However, this increase was not significant when compared to that observed under conditions emulating gingivitis.

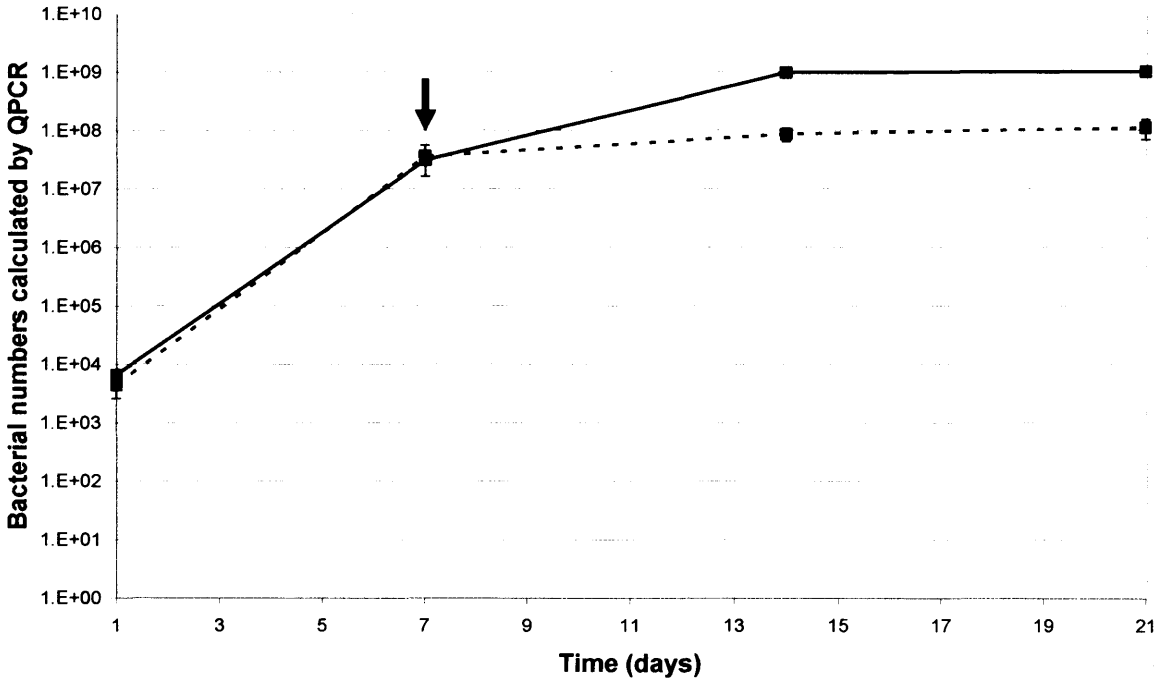

Figure 5.3.5: Total counts for *Prevotella* spp. determined by qPCR. Solid lines represent experiments where gingivitis conditions were implemented on day 7 (indicated by arrow); dashed lines represent experiments under conditions emulating health throughout. Error bars represent standard deviations ($n = 8$).

Under conditions emulating health, on day 7, the total counts for *Fusobacterium* spp. determined by qPCR ranged between 5.1 and 6.8 x 10^7 bacteria (Fig. 5.3.6). When conditions were switched to emulate gingivitis after day 7 the total counts for *Fusobacterium* spp. increased slightly, reaching numbers of 2.8 (+/- 0.4) x 10^8 by day 21. In experiments where conditions emulating health were maintained throughout the total counts for *Fusobacterium* spp. determined by qPCR continued to increase over

time, maximum numbers of 1.4 (+/- 0.6) x 10^8 being detected on day 21, meaning there was no real difference in numbers under conditions emulating health or gingivitis.

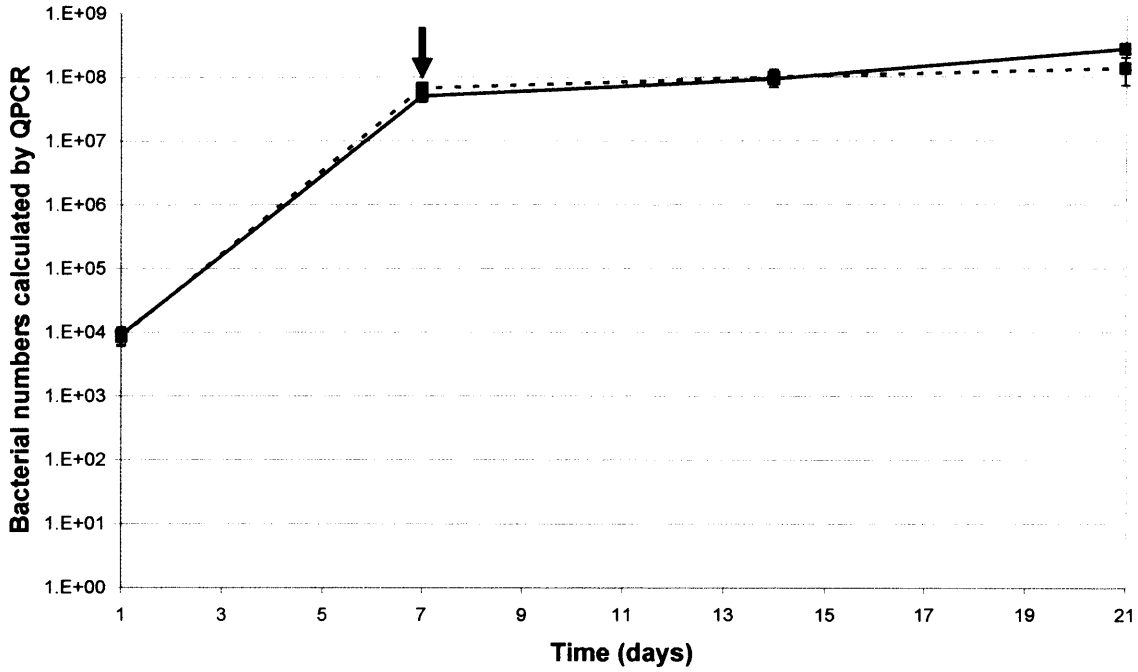

Figure 5.3.6: *Fusobacterium* spp. numbers calculated by qPCR. Solid lines represent experiments where gingivitis conditions were implemented on day 7 (indicated by arrow); dashed lines represent experiments under conditions emulating health throughout. Error bars represent standard deviations ($n = 8$).

Under conditions emulating health, on day 7, the number of *P. gingivalis* determined by qPCR ranged between 1.7 and 2.0 x 10^3 bacteria (Fig. 5.3.7). After day 7 when conditions were switched to emulate gingivitis the numbers of these organisms increased and by day 21 were found to be 2.4 (+/- 0.6) x 10^4, representing a significant increase ($P < 0.05$) in the number of *P. gingivalis* present. In experiments where conditions emulating health were maintained throughout the number of *P. gingivalis* determined by qPCR continued to increase over time, with a maximum number of 1.1

(+/- 0.6) x 10^4 detected on day 21. The numbers increased more rapidly and to higher levels under conditions emulating gingivitis.

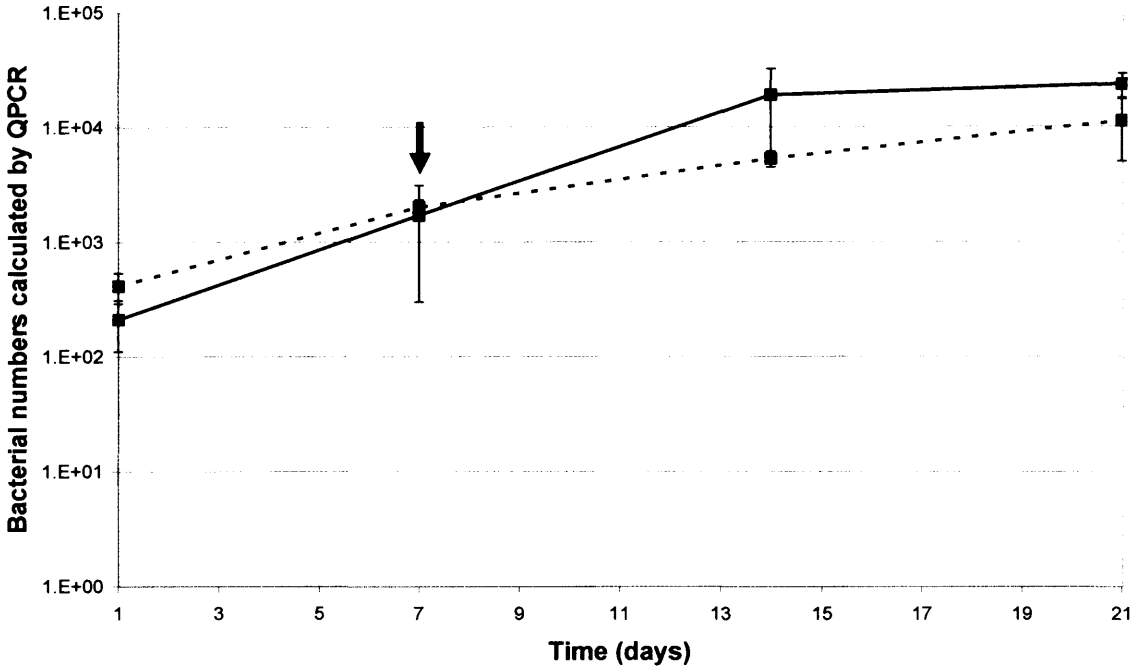

Figure 5.3.7: ***P. gingivalis*** **numbers calculated by qPCR.** Solid lines represent experiments where gingivitis conditions were implemented on day 7 (indicated by arrow); dashed lines represent experiments under conditions emulating health throughout. Error bars represent standard deviations (n = 8).

The estimation of the numbers of Gram-negative species present in the biofilm communities by culture did not correspond with the numbers estimated by qPCR (Fig. 5.3.8). The results of qPCR showed that *Prevotella* spp. were present in significant numbers in biofilm samples taken both before and after the induction of conditions emulating gingivitis, with the numbers increasing under conditions emulating gingivitis. *Fusobacterium* spp. were detected at high levels in samples taken both before and after induction of gingivitis conditions, however, in contrast to the *Prevotella* spp., the

numbers remained relatively stable under both conditions. *P. gingivalis* were detected in saliva, the inoculum and at low levels from microcosm plaque samples taken throughout each CDFF experiment (Fig. 5.3.7) with numbers increasing under conditions emulating gingivitis.

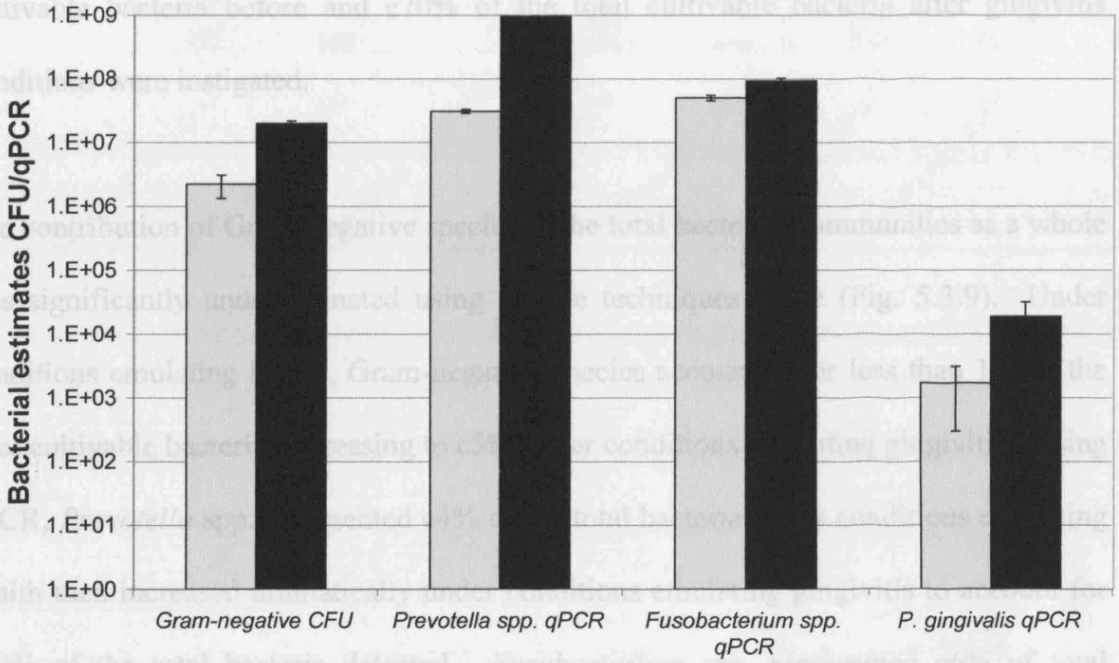

Figure 5.3.8: Numbers of Gram-negative bacteria calculated by culture (CFU) on CBA-GN and qPCR. ▪ biofilms grow under conditions emulating health; ■ represents biofilms grown under conditions emulating gingivitis. Error bars represent standard deviations (n = 8).

The contribution of different species to the community as a whole was different when assessed by culture or qPCR. Under conditions emulating health, *Streptococcus* spp. represented c32% of the total bacteria while under conditions emulating gingivitis they represented only c2% of the total bacteria detected using qPCR (Fig. 5.3.9). In contrast, using culture techniques, *Streptococcus* spp. represented c58% of the total cultivable

bacteria under conditions emulating health and c5% of the total cultivable bacteria under conditions emulating gingivitis. Under conditions emulating health *A. naeslundii* accounted for c2% of the total bacteria detected by qPCR. In contrast, once conditions were altered to emulate gingivitis *A. naeslundii* accounted for c37% of the total bacteria detected. Using culture techniques *Actinomyces* spp. accounted for c4% of the total cultivable bacteria before and c70% of the total cultivable bacteria after gingivitis conditions were instigated.

The contribution of Gram-negative species to the total bacterial communities as a whole was significantly underestimated using culture techniques alone (Fig. 5.3.9). Under conditions emulating health, Gram-negative species accounted for less than 1% of the total cultivable bacteria, increasing to c5% under conditions emulating gingivitis. Using qPCR, *Prevotella* spp. represented c4% of the total bacteria under conditions emulating health then increased dramatically under conditions emulating gingivitis to account for c49% of the total bacteria detected. *Fusobacterium* spp. represented c6% of total bacteria under conditions emulating health and c8% under gingivitis conditions and *P. gingivalis* represented a very small portion (less than c0.1%) of the total bacteria detected under either environmental condition.

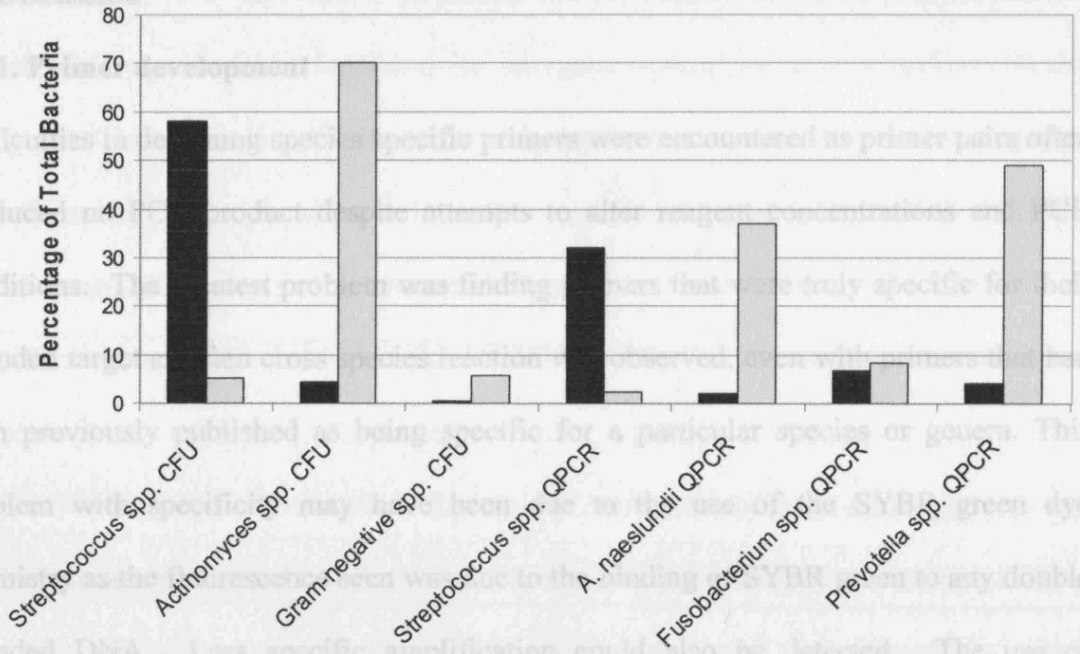

Figure 5.3.9: Different species/genera represented as a percentage of the total bacteria by culture and qPCR. ■, represents percentages in biofilms grown under conditions emulating health; ■, represents percentages in biofilms grown under conditions emulating gingivitis.

5.4 Discussion

5.4.1. Primer development

Difficulties in designing species specific primers were encountered as primer pairs often produced no PCR product despite attempts to alter reagent concentrations and PCR conditions. The greatest problem was finding primers that were truly specific for their intended target as often cross species reaction was observed, even with primers that had been previously published as being specific for a particular species or genera. This problem with specificity may have been due to the use of the SYBR green dye chemistry as the fluorescence seen was due to the binding of SYBR green to any double stranded DNA. Less specific amplification could also be detected. The use of fluorescent probes and beacons such as TaqMan probes may have been better but previous studies where primers have been developed for oral species for use with both TaqMan and SYBR green chemistries have shown no significant difference in terms of specificity, sensitivity and the quantity of product produced (Maeda *et al,* 2003).

As the region to be amplified with qPCR is so small, finding specific regions as targets for potential species-specific primers is more difficult. The 16S rRNA region was selected due to known regions of specificity that can be used for sequencing identification. As the 16S rRNA gene is present in all bacteria, usually in multiple copy numbers it was an ideal candidate for qPCR primer selection. The DNA sequences for the 16S rRNA gene are widely available from the BLAST database and are therefore a logical target for comparison. However, it may be that species specific 16S regions were not always suitable as the degree of similarity between some species in certain genera is too close to distinguish between species. For example, *Streptococcus* spp. from a particular group (e.g. mitis group) can not be reliably distinguished from each

other based on their 16S rRNA sequence. An alternative region to provide distinct sequences for species identification is the intergenic spacer region between the 16S and 23S genes. This region is thought to show higher degrees of variability between species from the same genera but attempts to use this region were also unsuccessful. Ideally a greater range of species and genera would have been assessed using qPCR but due to the difficulties mentioned above and the cost of the reagents this was not possible in this study.

5.4.2 Characterising oral biofilms associated with health and disease using qPCR

Microcosm plaques grown in the CDFF model have been characterised by direct amplification and cloning of 16S rRNA genes (Pratten *et al,* 2003a), revealing that only five species were detected by cloning that were not detected by culture, including *Prevotella* spp. and *F. nucleatum.* This indicated that most species grown in this model could be detected using culture or that more species could not be detected due to the inherent biases of cloning techniques. Similarly, using denaturing gradient gel electrophoresis (DGGE) to create genetic fingerprints of microcosm communities developed in the CDFF (McBain *et al,* 2003a), it was shown that few dominant bands were present, corresponding to a low species diversity. By identifying the cultivable flora by 16S rRNA gene sequencing in the previous chapter it was observed that a greater number of species from a wider range of genera were identified after changing the environmental conditions to emulate gingivitis. The aim of this part of study was to get a greater understanding of the flux in genera or species over time with quantitative PCR being the most advantageous method to do this. A major advantage of using qPCR as opposed to selective media for the enumeration of key oral species is the ability to differentiate between species of the same genera which are often hard to

distinguish on selective media without subculture and biochemical testing. This is particularly true for *A. naeslundii* and *A. viscosus* which are difficult to differentiate, even with biochemical tests (Hall *et al,* 1999).

The most significant observation from this study is the confirmation of trends observed for these species using culture with qPCR. The results of qPCR confirmed the trends observed previously in that the number of *Actinomyces* spp. (*A. naeslundii* in particular) increased as *Streptococcus* spp. decreased when the medium and atmospheric conditions were altered. Colony counts for *Actinomyces* spp. were higher on the selective agar used than counts of *A. naeslundii* using qPCR indicating that more species of *Actinomyces* were present in the biofilms. Indeed, a greater richness of *Actinomyces* spp. was identified by 16S rRNA gene sequencing in Chapter 4 with *A. viscosus*, *A. suismastidis* and *A. turicensis* also being identified.

Over time, the total bacterial numbers remained in a pseudo-steady state whether determined by culture or qPCR, implying that once communities had become established the bacterial numbers were controlled by spatial rather than nutritional limitations. Any fluxes in the populations appear to be related to the species proportions rather than total bacterial numbers. The total quantification by qPCR was always higher than those estimated by culture indicating that a certain portion of the population was not being accounted for by traditional culture techniques alone. The mean estimates of total bacteria calculated by qPCR with universal primers was 6.8×10^8 ($+/- 1.4 \times 10^8$) bacteria per biofilm whereas by colony counting the mean count was significantly less at 1.4×10^8 ($+/- 3.4 \times 10^7$) colony forming units per biofilm, which represented, on average, only 20% of the microbial population estimated by qPCR.

This may be due to the detection of uncultivable species by qPCR (Nadarkarni *et al,* 2002) and possibly due to cells being in a viable nonculturable state (Lleo *et al,* 1998). Additionally, within the nutrient limited biofilm environment, organisms with slower growth rates may have an advantage over faster growing organisms. For example, it has been shown that the slower growing cells in a single species community of *Escherichia coli* show an increased resistance to antibiotics (Evans *et al,* 1990) than faster growing members. Once removed from this environment their growth and survival may be significantly less than other members of the biofilm community and are thus underestimated as part of the microbial population by culture. Previous studies using qPCR to quantify total bacterial numbers in plaque samples have also observed this trend (Martin *et al,* 2002; Nadakarni *et al,* 2002). However, it cannot be assumed that this difference is entirely due to uncultivable bacteria. When quantifying the total bacteria using universal primers based on the 16S rRNA gene the total ribosomal RNA present in the sample is being quantified (Horz *et al,* 2005). The rRNA copy number is not uniform for all bacteria, varying between 1 and 15 copies per cell for different species (Farrelly *et al,* 1995; Horz *et al,* 2005; Nadakarni *et al,* 2002) and it is therefore possible that this could account for some of the shortfall between total counts obtained by culture and bacterial numbers assessed by qPCR. An attempt to minimise the influence of varying copy number was employed by using DNA extracted from a variety of oral species to create the standard curves for the calculation of total bacterial numbers.

Prevotella spp. accounted for approximately 49% of the total bacteria detected after the induction of gingivitis conditions but were rarely isolated using culture techniques, *Prevotella veroralis* being the only species isolated from saliva and from biofilm

samples after the induction of gingivitis conditions (Chapter 4). Martin *et al,* (2002) detected significantly higher counts for *Prevotella* spp. by qPCR, using the same primers used in this study, than were detected using culture techniques, even when selective media for *Prevotella* spp. were used. The numbers of *Prevotella* spp. also increased under conditions emulating gingivitis, indeed this genus is commonly isolated from dental plaque after the development of periodontal diseases (Lie *et al,* 2001; Ximenez-Fyvie *et al,* 2000b).

With qPCR it was also possible to assess the proportion of other Gram-negative species in the microbial community. While *Fusobacterium* spp. were significant members of the biofilm communities their numbers did not really increase under conditions emulating gingivitis even though these organisms are often linked to periodontal disease. This may be due to *Fusobacterium* spp. lacking the endopeptidase activity required to break down large proteinaceous nutrients present in GCF. *F. nucleatum* do possess aminopeptidase activity (Rogers *et al,* 1998) and can thus utilise protein derived nutrients (e.g. amino acids and oligo peptides) by linking to proteolytic organisms such as *P. gingivalis* (Kolenbrander *et al,* 2002). *P. gingivalis* was detected in the pooled saliva and the inoculum at levels comparable to the other species but did not form a significant part of the microcosm communities sampled. Supragingival plaque communities associated with health do not usually contain high numbers of *P. gingivalis* (Lau *et al,* 2004), however the numbers often increase with the development of periodontal diseases (Ximenez-Fyvie *et al,* 2000a) and tend to form more significant members of the subgingival plaque community (van Winkelhoff *et al,* 2002). The numbers detected by qPCR did increase ten-fold under conditions emulating gingivitis but still represented a small proportion of the community, indicating that numbers were

not high enough initially for *P. gingivalis* to become a key organism in these communities, even under conditions that could favour their growth. Serum is a constituent of GCF and had been included in the medium used to emulate gingivitis, it has also been shown to enhance the growth of *Prevotella* spp. and *P. gingivalis* (ter Steeg *et al,* 1987).

5.4.3 Conclusions

qPCR revealed that *A. naeslundii*, *Prevotella* spp. and *P. gingivalis* all increased under conditions emulating gingivitis. Using qPCR it was also revealed that Gram-negative species were dramatically underestimated by the culture techniques used in the previous chapter. Furthermore, the total bacterial counts enumerated by culture were underestimated by approximately 80% compared to the total numbers estimated by qPCR, highlighting the benefits of using this approach as opposed to traditional culture. The use of qPCR can be considered as an effective method for rapid assessment of changes in microbial communities associated with health and disease, making it particularly useful for studying how microbial communities would be affected by the presence of antimicrobial agents which will be discussed in the next chapter.

CHAPTER 6

Development of antibacterial and antiplaque coatings on hydroxyapatite and their effect on the development of dental plaque microcosm communities

6.1 Introduction

Plaque-related diseases such as gingivitis, periodontitis and peri-implantitis are associated with inadequate dental hygiene. This allows the accumulation of plaque and the proliferation of bacteria which will encourage gingival inflammation, tissue damage and loss of attachment or in the case of peri-implantitis rejection of the dental implant. Treatments for plaque-related diseases are generally focused on reducing the amount of accumulated plaque by mechanical or chemical means. The aim with such treatments is not to completely eradicate the microbial population but to maintain species and levels of colonisation which are conducive for the maintenance of gingival health. In cases where mechanical measures of plaque removal are inadequate, for example, due to lack of compliance or are not possible due to recent oral surgery, the use of antimicrobial and antiplaque agents is beneficial. The use of antimicrobials and antiplaque agents has been shown to be effective against the development of periodontal diseases (Jenkins *et al,* 1994). However, for such agents to be effective they need to remain in the oral cavity for significant amounts of time.

Antibacterial agents are most effective against the accumulation of plaque rather than reducing bacterial numbers in an already established biofilm (Pratten *et al*, 1998a; Eley, 1999). In general, bacteria growing as part of a biofilm are more resistant to antimicrobial agents than their planktonic counterparts (Evans *et al*, 1990b; Duguid *et al*, 1992; Ashby *et al*, 1994; Aaron *et al*, 2002). In order for an agent to be effective against biofilms up to 500 times the MIC determined for planktonic bacteria may be required (Larsen & Fiehn, 1996). For example, for metronidazole to be effective against *P. gingivalis* biofilms 160 times the MIC for planktonic cells is required (Wright *et al,* 1997).

When preventing periodontal disease the main disadvantage with standard delivery methods for therapeutic agents such as toothpastes and mouthwashes is low substantivity at the target site i.e. the agents are not maintained at a concentration where they could have significant antibacterial effect and low levels of penetration into the biofilm due to the issues discussed in Chapter 1 (Section 1.5.2). The use of local delivery systems that prolong the release of active agents into the mouth are advantageous in the treatment of periodontal diseases (Steinberg & Friedman, 1999) and are thought to be one of the most cost-effective approaches to the treatment of these diseases (De Lissovoy *et al,* 1999). Incorporation of these agents into dental materials can provide sustained release into the oral cavity, reducing levels of plaque accumulation. Traditional agents for such use are chlorhexidine and tetracycline which have a broad spectrum action against oral species (Mandel, 1988; Chopra & Roberts, 2001). Alternative agents for bacterial control in the oral cavity include metal ions such as silver ions which are more active against prokaryotic cells than eukaryotic cells (Marino *et al,* 1974; Berger *et al*, 1976) and are more effective against Gram-negative species (Kawahara *et al,* 2000) making them ideal candidates for use in the oral cavity.

The previous chapters have described how altering key environmental parameters can change the microbial populations of *in vitro* oral biofilms. The aim of this chapter was to examine the effect of sustained release of tetracycline, chlorhexidine and silver ions from hydroxyapatite discs on the development of dental plaque microcosms and to examine how key species and genera were differentially affected by these agents using qPCR.

6.2 Methods

6.2.1 Coating Hydroxyapatite (HA) discs with Tetracycline Hydrochloride (TCH) and Chlorhexidine diacetate (CHX)

Stock solutions of each agent in sterile deionised water were made up in amber bottles to prevent degradation by exposure to light. For TCH (Sigma) solutions of 1, 5 and 10 mg/ml were made. For CHX (Sigma) solutions of 5, 10 and 15 mg/ml were made. Individual HA discs were then immersed in 5 ml of stock solution in sterile plastic bijou bottles kept inside amber bottles to prevent exposure to light. This was repeated in triplicate for each agent at each concentration used.

6.2.1.1Characterising adsorption and release profiles for TCH and CHX

6.2.1.1.1 Calibration curves

In order to characterise the amount of adsorption and release for each agent it was first necessary to create a calibration curve from solutions of a known concentration of each agent within the range of detection. For each agent a calibration curve was created from solutions of concentrations varying from 0.01 to 0.1 mg/ml.

6.2.1.1.1.1 TCH calibration curve

The UV absorbance was measured (Unicam UV500 UV/Visible spectrophotometer; Thermospectronic, Rochester, NY, USA) in the range of 200 to 600 nm for TCH solutions of varying concentrations (Fig. 6.2.1).

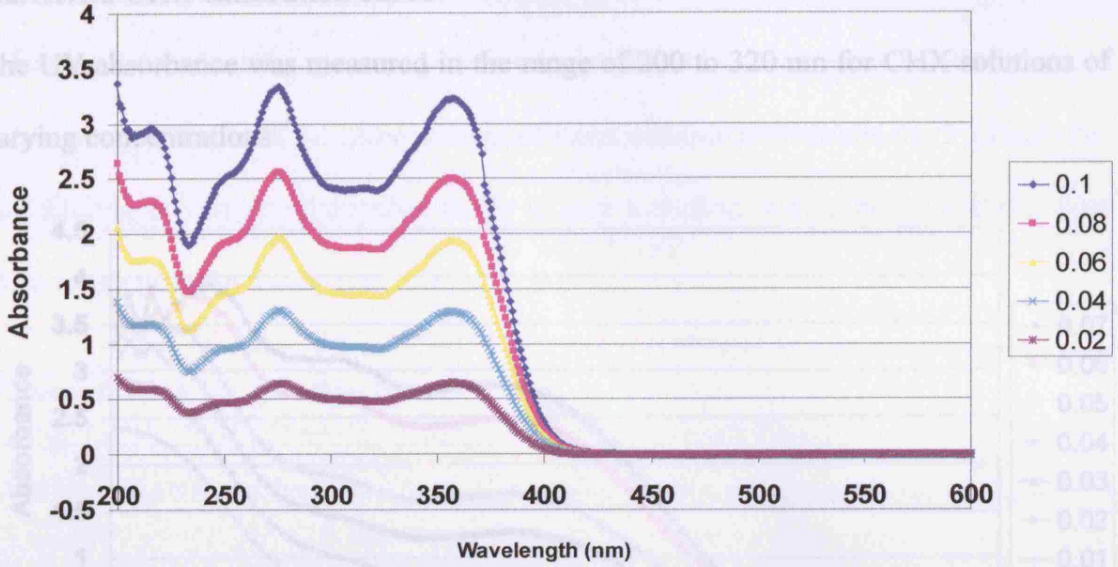

Figure 6.2.1: UV absorbance scans for TCH (mg ml $^{-1}$) solutions.

The absorbance value at 357 nm was used to create a calibration curve for TCH (Fig. 6.2.2).

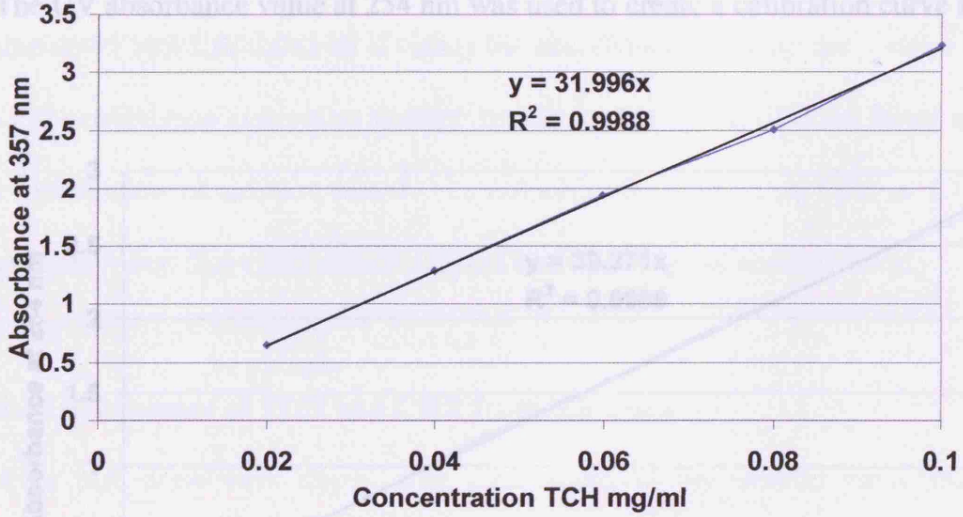

Figure 6.2.2: Calibration curve for TCH.

6.2.1.1.1.2 CHX calibration curve

The UV absorbance was measured in the range of 200 to 320 nm for CHX solutions of varying concentrations

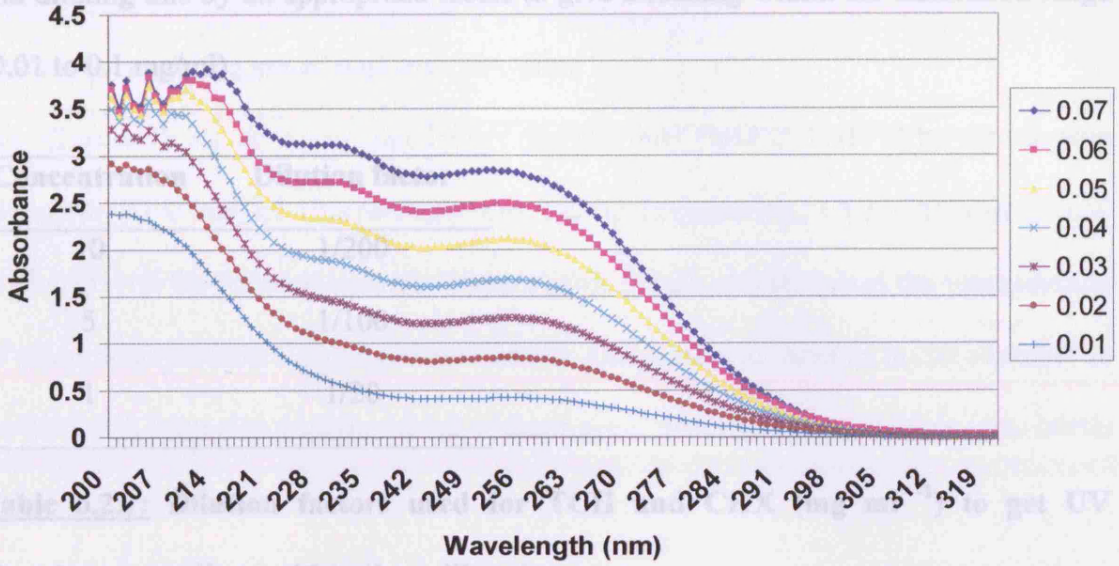

Figure 6.2.3: UV absorbance scans for CHX solutions.

The UV absorbance value at 254 nm was used to create a calibration curve for CHX.

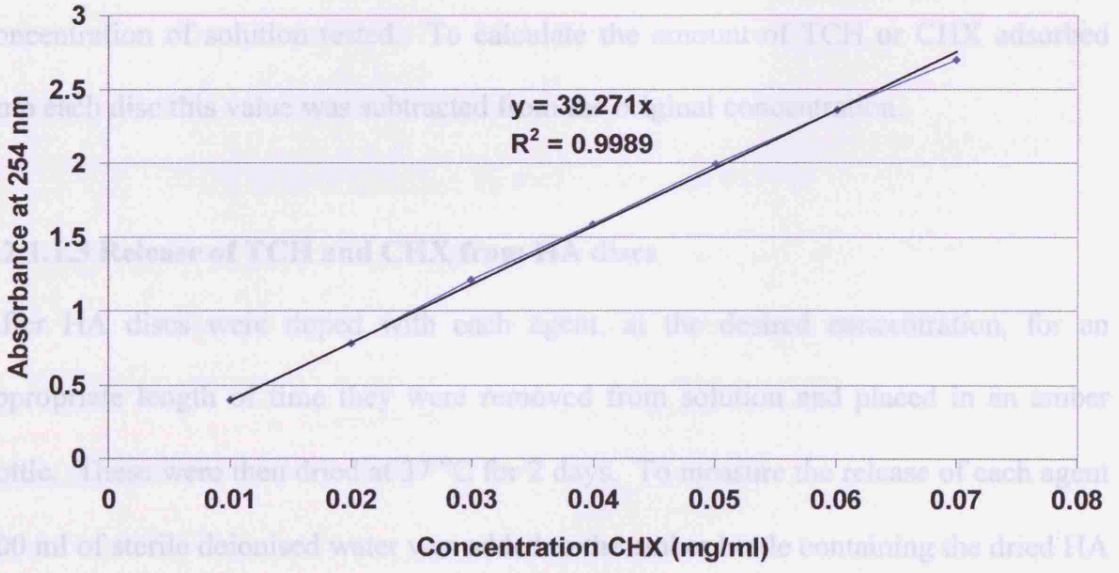

Figure 6.2.4: Calibration curve for CHX.

6.2.1.1.2 Adsorption of TCH and CHX onto HA

Adsorption values at the set time points were obtained by removing a small amount of solution (replacing with the same amount of stock solution to maintain the 5 ml volume) and diluting this by an appropriate factor to give a reading within the calibration range (0.01 to 0.1 mg/ml).

Concentration	Dilution factor
10	1/200
5	1/100
1	1/20

Table 6.2.1: Dilution factors used for TCH and CHX (mg ml $^{-1}$) to get UV absorbance readings within the calibration range.

The amount of TCH or CHX present in the solution in which each HA disc was immersed was calculated by dividing the absorbance value by the *y* value for the slope of the calibration curve then multiplying this value by the dilution factor used for each concentration of solution tested. To calculate the amount of TCH or CHX adsorbed onto each disc this value was subtracted from the original concentration.

6.2.1.1.3 Release of TCH and CHX from HA discs

After HA discs were doped with each agent, at the desired concentration, for an appropriate length of time they were removed from solution and placed in an amber bottle. These were then dried at 37 °C for 2 days. To measure the release of each agent 100 ml of sterile deionised water was added to the amber bottle containing the dried HA disc. The amount of agent released into this solution was then measured by removing 3

ml of this solution at set time points, measuring the UV absorbance at the appropriate wavelength, then returning this solution back to the bijou to maintain the volume at 100 ml.

6.2.2 Incorporating silver ions into HA discs

HA discs with silver incorporated were pressed and fired at 1300°C by Dr. George Georgiou (Division of Biomaterials and Tissue Engineering, UCL Eastman Dental Institute) with the following compositions (Table 6.2.2). All discs had the same amount of silver incorporated. A composition of HA and glass was used as initial attempts to produce HA with silver incorporated without glass resulted in discs which were brittle and easily damaged.

	Composition
10 %	**HA + 10 wt% CP Ag glass**
	(Ca = 30 mol%; P_2O_5 = 50 mol%; Ag = 20 mol%)
15 %	**HA + 15 wt% CP Ag glass**
	(Ca = 30 mol%; P_2O_5 = 50 mol%; Ag = 20 mol%)
20 %	**HA + 20 wt% CP Ag glass**
	(Ca = 30 mol%; P_2O_5 = 50 mol%; Ag = 20 mol%)
Control	**HA + 2.5 wt % CNP glass**
	(Ca = 30 mol%; P_2O_5 = 50 mol%; Ag = 0 mol%)

Table 6.2.2: Compositions of Ag-HA used.

6.2.3 Antibacterial effect of TCH-HA and CHX-HA

To ensure that there was an antibacterial effect of each agent standard disc diffusion assays were carried out with TCH-HA and CHX-HA discs on confluent growth of *S. sanguinis* NCTC 7863 (British Society for Antimicrobial Chemotherapy disc diffusion method).

The effect of TCH-HA and CHX-HA discs on the growth of planktonic cultures of *S. sanguinis* NCTC 7863 was also measured. This was done by adding 1 ml of overnight culture to 9 ml of fresh BHI broth in a sterile universal bottle containing a TCH-HA or CHX-HA disc, placed in a shaking incubator at 37° C, then measuring the growth of these populations over time (4 and 24 h) by serial dilutions on FAA plates which were incubated anaerobically at 37° C for 4 days.

6.2.4 Effect of therapeutic agents on microcosm communities

6.2.4.1. CDFF setup

6.2.4.1.1 TCH and CHX doped HA discs

The CDFFs were set up as described in chapter 2 except the TCH or CHX-doped HA discs were not put in before autoclaving as TCH and CHX would be degraded. After autoclaving, whilst still hot the doped HA discs were put in to the CDFF and recessed to 600 μm inside a flow cabinet to keep the CDFF as sterile as possible.

6.2.4.1.2 HA discs with Ag ions incorporated

The CDFFs were set up as described in chapter 2 with the Ag ion incorporated discs inside but were sterilised by heating in an oven at 160°C for 1 hour instead of autoclaving.

6.2.4.2 Generation of biofilms

Microcosm dental plaque biofilm communities were grown in the CDFF as described in Chapter 2, except two fermenters were inoculated simultaneously with the same inoculum, for each agent. One fermenter was set-up to grow organisms under conditions emulating health for 7 days, and then switched to conditions emulating gingivitis. The second fermenter was set-up for conditions emulating gingivitis from day one.

6.2.4.3 Analysis of biofilms

6.2.4.3.1 Analysis of microcosm communities using qPCR

qPCR assays were performed using primers for *Prevotella* spp., *Fusobacterium* spp., *Streptococcus* spp., *P. gingivalis*, *A. naeslundii* and universal (total bacteria) as described in Chapter 2 and 5.

6.3 Results

6.3.1 Adsorption and release of agents on HA

6.3.1.1 TCH adsorption onto HA

The adsorption of TCH onto the HA discs is shown in Figure 6.3.1. Adsorption levels stabilized after 24 hours for all concentrations tested, after which time no more adsorption occurred.

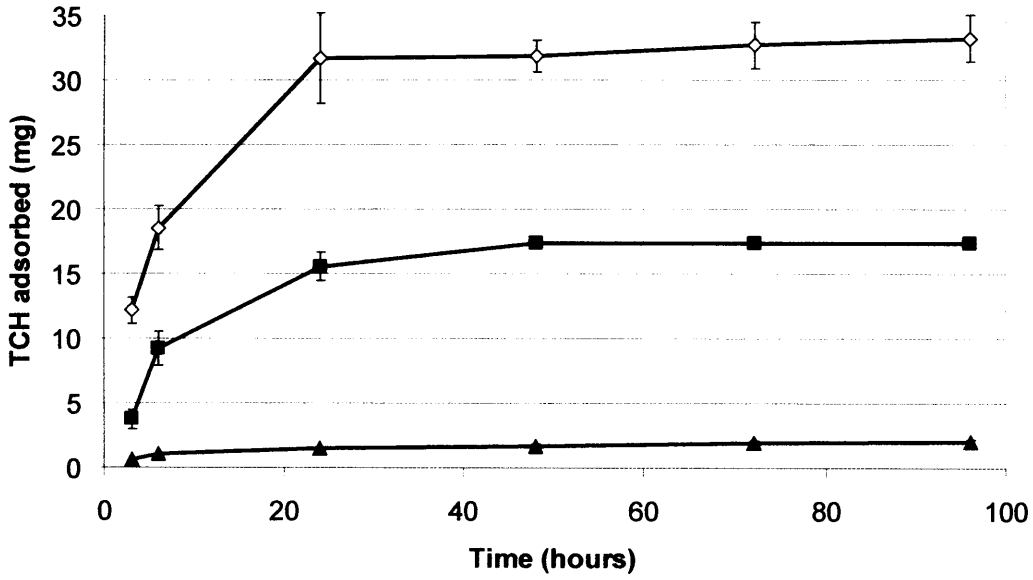

Figure 6.3.1: Tetracycline adsorption onto hydroxyapatite. ◊, represents 10, ■ represents 5, ▲ represents1 mg ml $^{-1}$ TCH solution. Error bars represent the standard deviation ($n = 3$).

The total amount of TCH adsorbed onto each HA disc was 33.2, 17.4 and 2.05 mg for the 10, 5 and 1 mg ml $^{-1}$ TCH solutions respectively. The highest levels of adsorption were observed for discs immersed in TCH solutions of the highest concentration adsorbed from solution after 96 hours.

6.3.1.2 TCH release from HA

The release of TCH from HA discs is shown in Figure 6.3.2. After 48 hours the release of TCH stabilized for all concentrations. The total amount of TCH released from each disc was 6.9, 7.0 and 1.5 mg for the 10, 5 and 1 mg ml $^{-1}$ TCH solutions respectively.

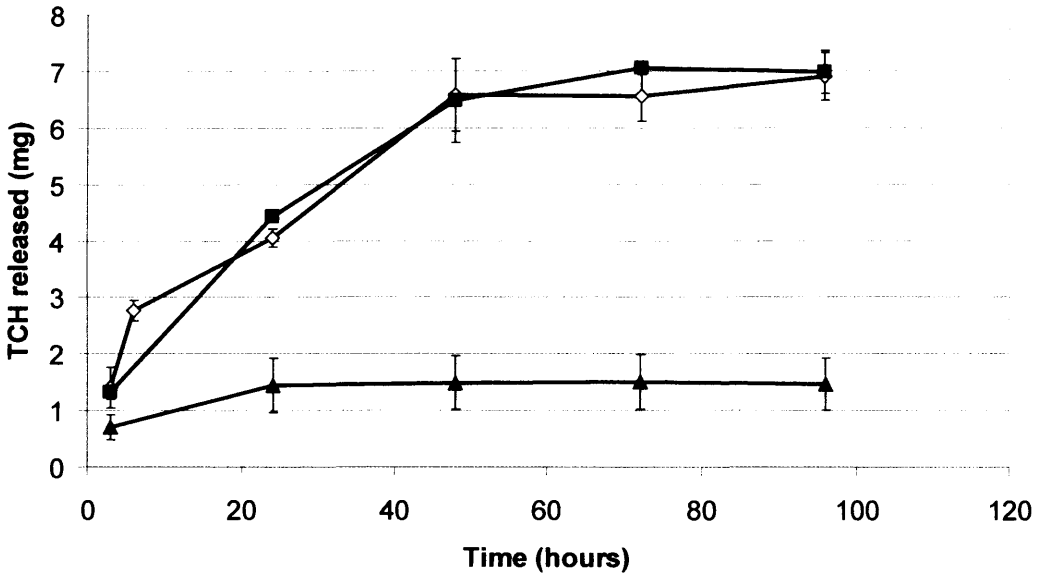

Figure 6.3.2: Tetracycline release from hydroxyapatite. ◊ represents 10, ■ represents 5, ▲ represents1 mg ml $^{-1}$ TCH solution. Error bars represent the standard deviation (n = 3).

No difference was observed in the amount of TCH release from the HA disc immersed in TCH solutions of 10 and 5 mg ml $^{-1}$ TCH even though more TCH was adsorbed onto the discs immersed in the 10 mg ml $^{-1}$ TCH solution. The amount of TCH released from each HA disc represented 20.8, 40.2 and 73.2 % of the total amount adsorbed for the 10, 5 and 1 mg ml $^{-1}$ TCH solutions respectively. This indicates that TCH was more efficiently released from HA discs which had adsorbed a lower amount of TCH.

6.3.1.3 CHX adsorption on HA

The adsorption of CHX onto the HA discs is shown in Figure 6.3.3. The pattern of adsorption to hydroxyapatite was different to that of TCH. The amount of CHX adsorbed had not stabilized after 96 hours for any concentration.

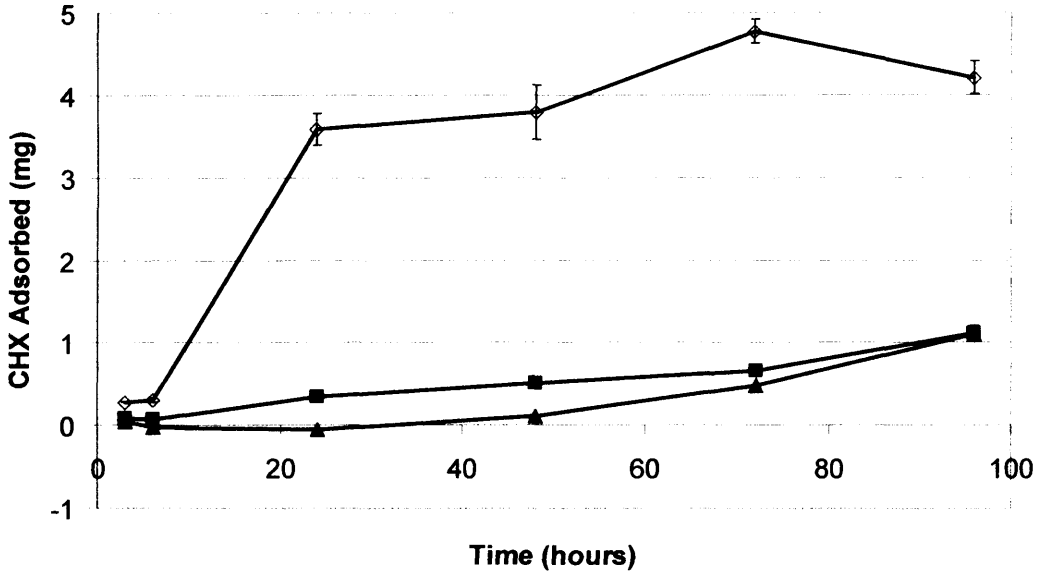

Figure 6.3.3: Chlorhexidine adsorption onto hydroxyapatite. ◊ represents 10, ■ represents 5, ▲ represents1 mg ml $^{-1}$ CHX solution. Error bars represent the standard deviation (n = 3).

The total amount of CHX adsorbed onto each HA disc was 4.21, 1.11 and 1.10 mg for the 10, 5 and 1 mg ml $^{-1}$ CHX solutions respectively. The amount of CHX adsorbed onto HA was much less than the amount of TCH adsorbed.

6.3.1.4 CHX release from HA

The amount of CHX released could not be measured accurately as levels of release were very low and thus were out of the range of calibration.

6.3.2 Antibacterial effect of doped HA discs

Before attempting microcosm experiments it was first necessary to determine whether the coating of HA discs with TCH and CHX was having any antibacterial effect on confluent growth of *S. sanguinis* on FAA plates. A zone of inhibition around the discs was observed for both agents at all concentrations.

6.3.2.1 Effect of TCH-HA on planktonic *S. sanguinis* growth

TCH-HA discs significantly reduced ($P < 0.05$) the growth of *S. sanguinis* after 4 hours at all concentrations. After 24 hours this growth was reduced further representing a greater than 99% inhibition at all concentrations of TCH used.

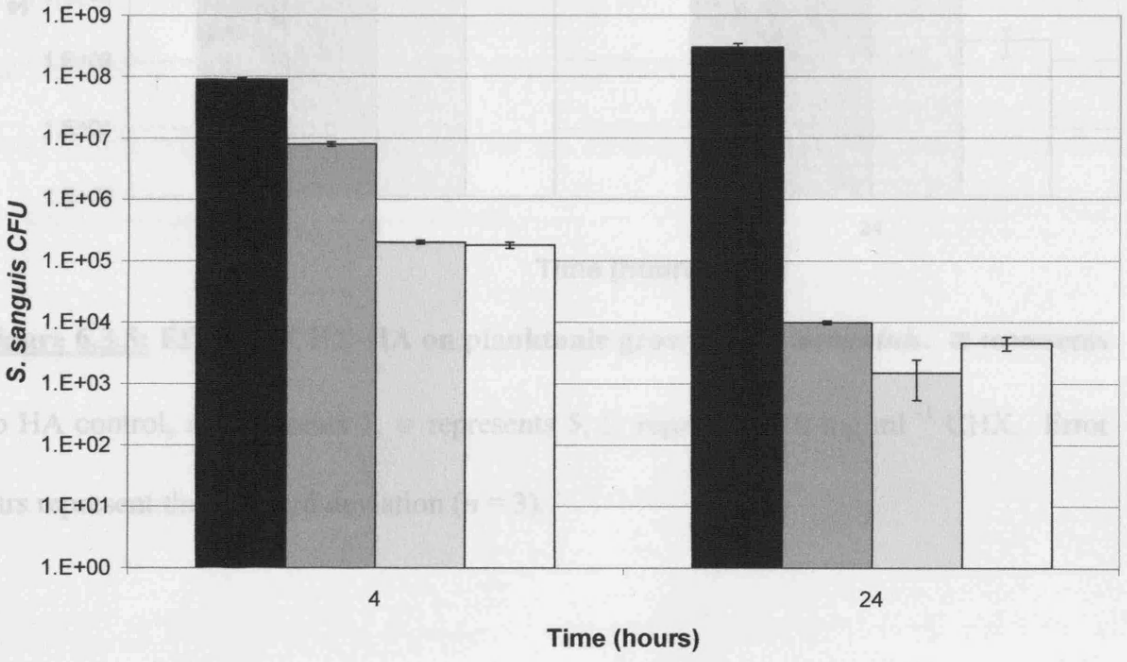

Figure 6.3.4: Effect of TCH-HA on planktonic growth of *S. sanguinis*. ■ represents no HA control, ■ represents 1, ■ represents 5, □ represents 10 mg ml $^{-1}$ TCH. Error bars represent the standard deviation ($n = 3$).

6.3.2.2 Effect of CHX-HA on *S. sanguinis* growth

After 4 hours of incubation with CHX-HA there was a significant reduction ($P < 0.05$) in growth of planktonic *S. sanguinis* for discs coated with the 5 and 10 mg ml $^{-1}$ CHX solutions. After 24 hours this growth was reduced further for the discs coated in the 5 and 10 mg ml $^{-1}$ CHX solutions, representing a greater than 99% inhibition at these concentrations.

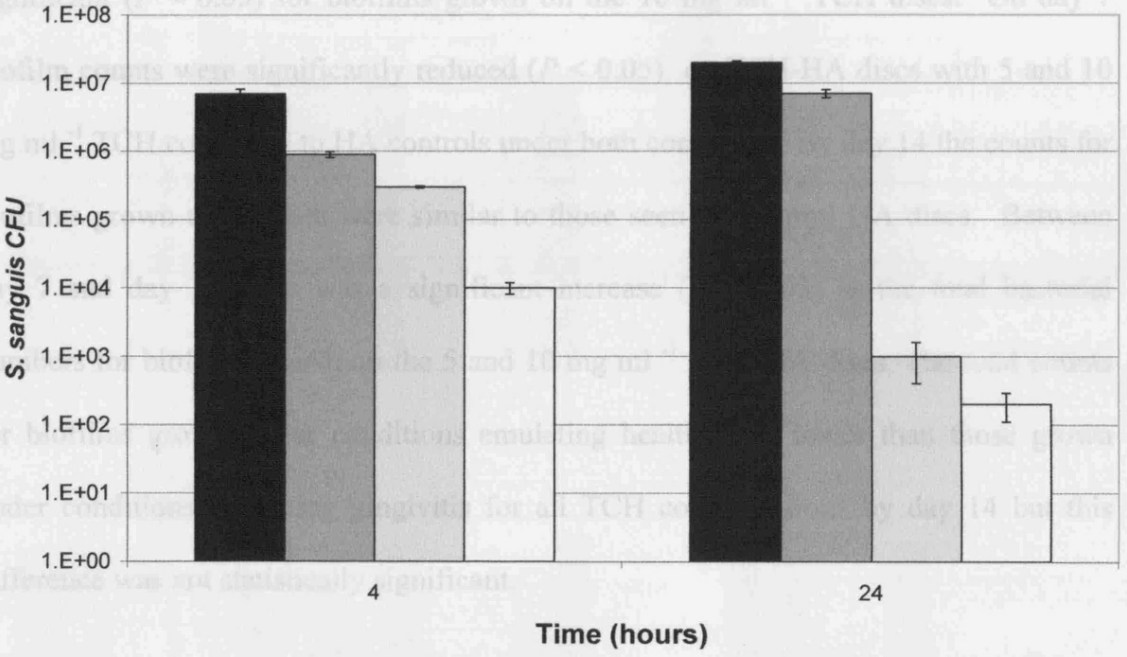

Figure 6.3.5: Effect of CHX-HA on planktonic growth of *S. sanguinis*. ■ represents no HA control, ■ represents 1, ■ represents 5, □ represents 10 mg ml $^{-1}$ CHX. Error bars represent the standard deviation ($n = 3$).

6.3.3 Analysis of microcosm communities exposed to different therapeutic agents

6.3.3.1 Tetracycline

6.3.3.1.1 Effect of TCH-HA on total bacterial counts by culture

Figure 6.3.6 shows the total bacterial counts by culture for TCH-HA discs. On day one of biofilm development reduced numbers of bacteria were observed from biofilms grown on TCH-HA discs with 5 and 10 mg ml $^{-1}$ TCH, but this reduction was only significant ($P < 0.05$) for biofilms grown on the 10 mg ml $^{-1}$ TCH discs. On day 7 biofilm counts were significantly reduced ($P < 0.05$) on TCH-HA discs with 5 and 10 mg ml $^{-1}$ TCH compared to HA controls under both conditions. By day 14 the counts for biofilms grown under both were similar to those seen for control HA discs. Between day 7 and day 14 there was a significant increase ($P < 0.05$) in the total bacterial numbers for biofilms grown on the 5 and 10 mg ml $^{-1}$ TCH-HA discs. The total counts for biofilms grown under conditions emulating health were lower than those grown under conditions emulating gingivitis for all TCH concentrations by day 14 but this difference was not statistically significant.

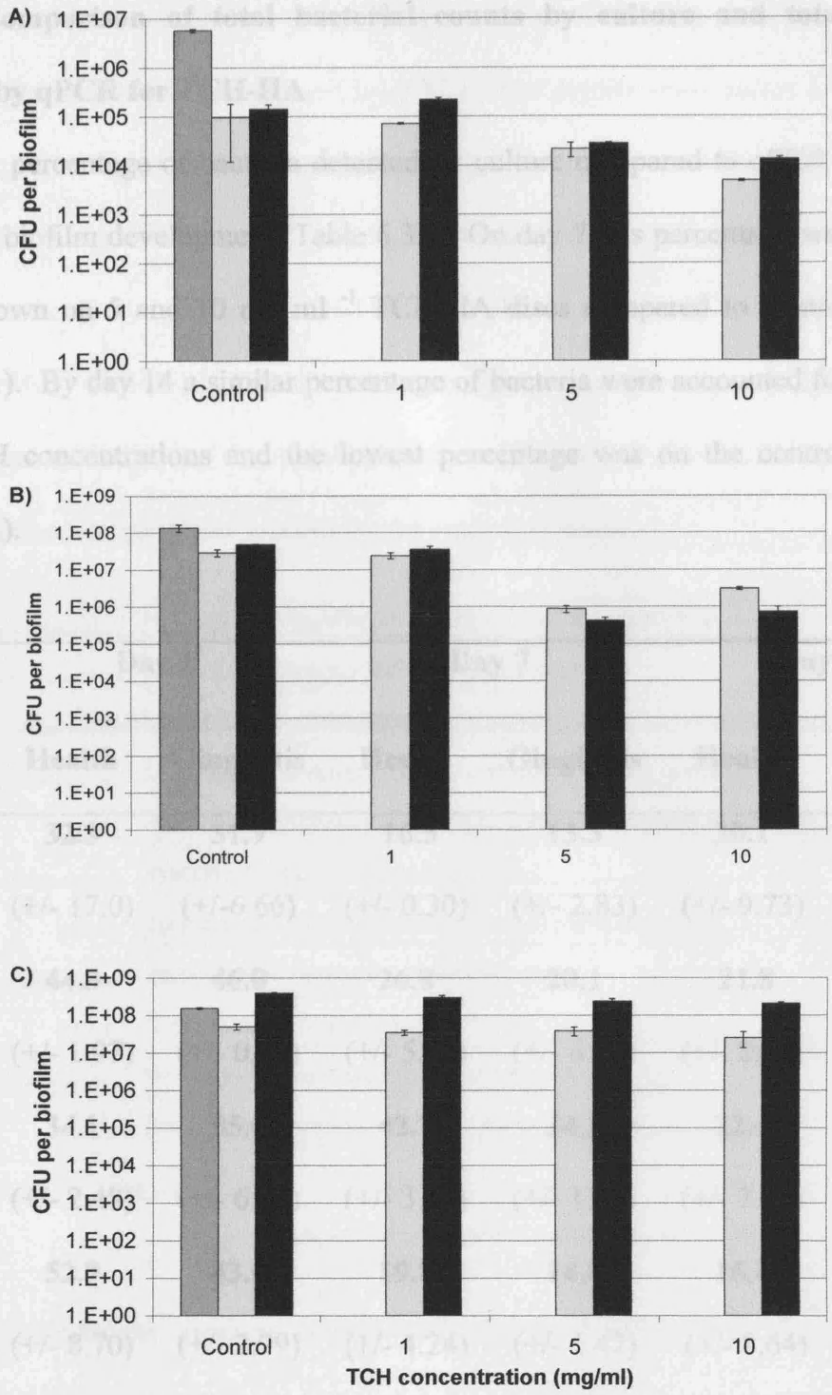

Figure 6.3.6: Total anaerobe counts on FAA under conditions emulating health or gingivitis for biofilms grown on TCH-HA. A) represents day 1, B) represents day 7, C) represents day 14 of biofilm development; ■ represents fermenter under conditions emulating health, ■ represents fermenter under conditions emulating gingivitis, ■ represents control from fermenter containing HA discs only. Error bars represent the standard deviations ($n = 6$).

6.3.3.1.2 Comparison of total bacterial counts by culture and total numbers calculated by qPCR for TCH-HA

The highest percentage of bacteria detected by culture compared to qPCR overall was on day 1 of biofilm development (Table 6.3.1). On day 7 this percentage was higher for biofilms grown on 5 and 10 mg ml $^{-1}$ TCH-HA discs compared to control HA discs (Table 6.3.1). By day 14 a similar percentage of bacteria were accounted for by culture for all TCH concentrations and the lowest percentage was on the control HA discs (Table 6.3.1).

TCH (mg/ml)	Day 1		Day 7		Day 14	
	Health	Gingivitis	Health	Gingivitis	Health	Gingivitis
1	32.3	31.9	16.5	13.3	20.1	24.4
	(+/- 17.0)	(+/-6.66)	(+/- 0.30)	(+/- 2.83)	(+/- 9.73)	(+/- 6.94)
5	44.3	46.0	26.8	20.1	21.8	17.2
	(+/- 1.97)	(+/- 0.61)	(+/- 5.66)	(+/- 4.19)	(+/- 2.46)	(+/- 2.41)
10	34.1	35.0	42.7	34.3	22.4	19.5
	(+/- 2.48)	(+/- 6.20)	(+/- 3.83)	(+/- 13.0)	(+/- 7.46)	(+/- 3.30)
Control	52.9	43.5	19.8	14.8	15.4	10.0
	(+/- 8.70)	(+/- 2.99)	(+/- 4.24)	(+/- 1.47)	(+/- 8.64)	(+/- 5.61)

Table 6.3.1: Total bacterial counts (CFUs) expressed as a percentage of the total bacterial numbers calculated by qPCR on TCH-HA. Data are means and standard deviations ($n = 6$).

6.3.3.1.3 Microbial composition of biofilms grown on TCH-HA

The total bacterial numbers calculated by qPCR were significantly lower ($P < 0.05$) for biofilms grown under conditions emulating gingivitis on discs coated with 5 and 10 mg ml $^{-1}$ concentrations of TCH (Figure 6.3.7) compared to controls. *Streptococcus* spp. numbers calculated by qPCR on TCH-HA were also significantly lower ($P < 0.05$) for biofilms grown under conditions emulating gingivitis for 5 and 10 mg ml $^{-1}$ concentrations of TCH. *A. naeslundii*, *Fusobacterium* and *Prevotella* spp. were not detected in these communities, except for on control HA discs.

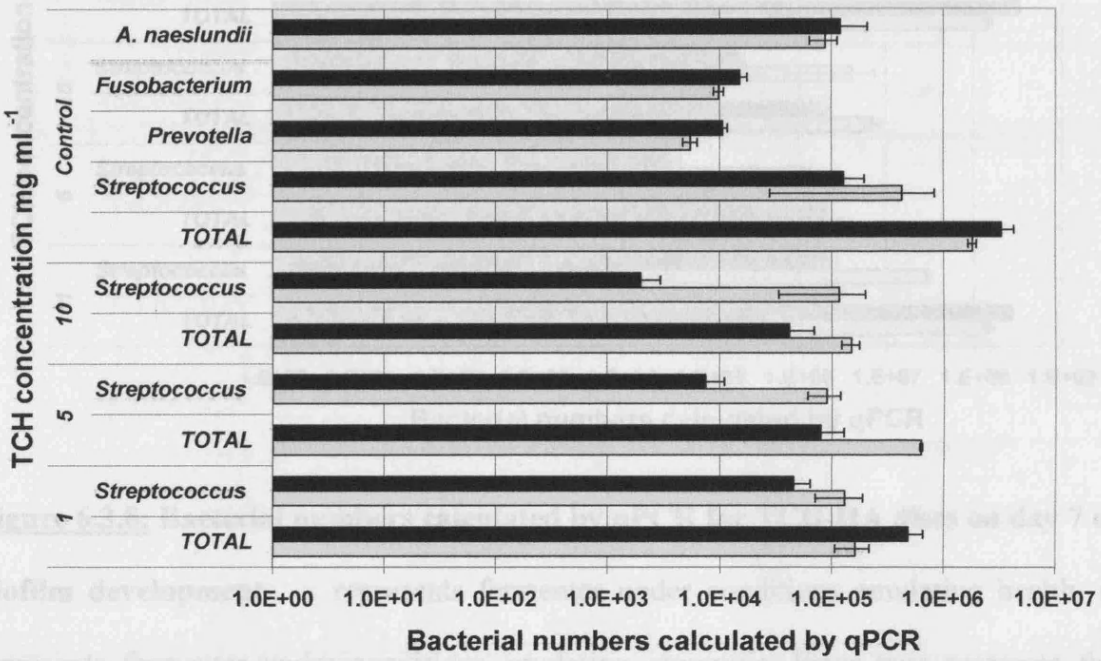

Figure 6.3.7: Bacterial numbers calculated by qPCR for TCH-HA discs on day 1 of biofilm development. ▪ represents fermenter under conditions emulating health, ■ represents fermenter under conditions emulating gingivitis. Error bars represent the standard deviations ($n = 6$).

By day 7 there was no difference in the total bacterial numbers for controls and the lowest concentration of TCH. For 5 and 10 mg ml $^{-1}$ TCH there was still a significant reduction ($P < 0.05$) in total bacteria (Figure 6.3.8). *Streptococcus* spp. numbers were lower for biofilms grown under conditions emulating gingivitis, except for on control HA discs.

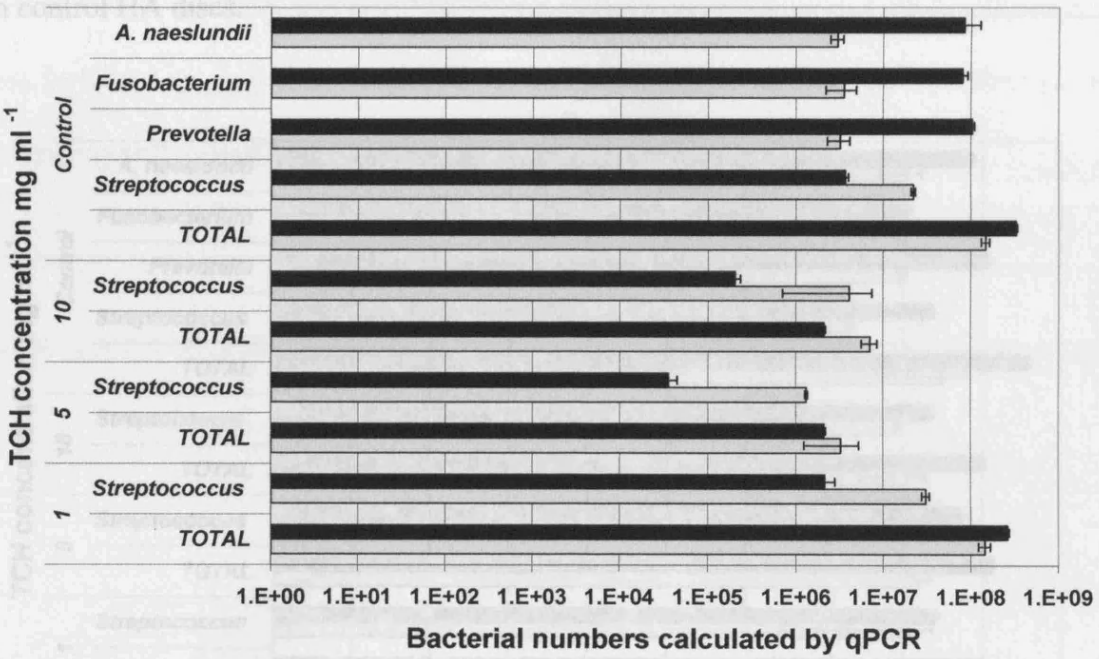

Figure 6.3.8: Bacterial numbers calculated by qPCR for TCH-HA discs on day 7 of biofilm development. ■ represents fermenter under conditions emulating health, ■ represents fermenter under conditions emulating gingivitis. Error bars represent the standard deviations ($n = 6$).

By day 14 the total bacterial numbers were similar for all concentrations of TCH, with biofilms grown under conditions emulating gingivitis having higher total numbers (Figure 6.3.9) but this difference was not statistically significant. *Streptococcus* spp. appeared to be the dominant genera under all conditions. Quantitative PCR for *A. naeslundii*, *Prevotella* and *Fusobacterium* spp. did not detect these species, except for on control HA discs.

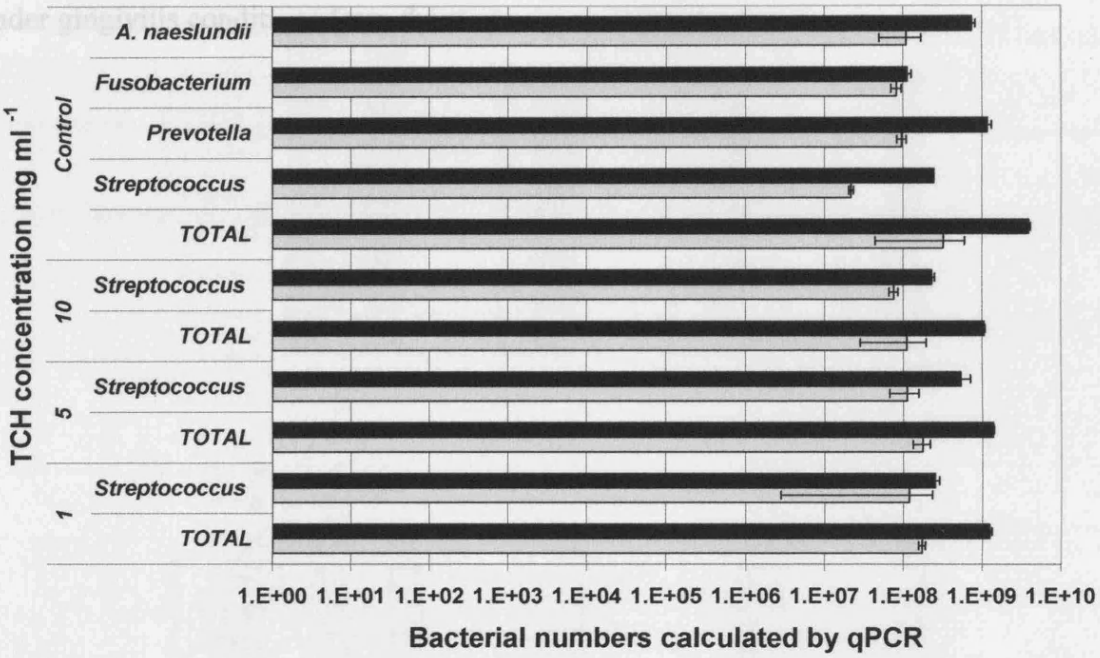

Figure 6.3.9: Bacterial numbers calculated by qPCR for TCH-HA discs on day 14 of biofilm development. ▪ represents fermenter under conditions emulating health, then switched to conditions emulating gingivitis on day 7, ■ represents fermenter under conditions emulating gingivitis. Error bars represent the standard deviations ($n = 6$).

6.3.3.2 Chlorhexidine

6.3.3.2.1 Effect of CHX-HA on total bacterial counts by culture

The total bacterial counts were significantly reduced ($P < 0.05$) on day 1 for CHX-HA discs compared to controls (Figure 6.3.10). By day 7 counts were the same for CHX-HA discs and control HA discs. By day 14 total counts were similar for all concentrations of CHX and biofilms grown under conditions emulating health initially then switched to gingivitis conditions on day 7 had higher counts than biofilms grown under gingivitis conditions from the start.

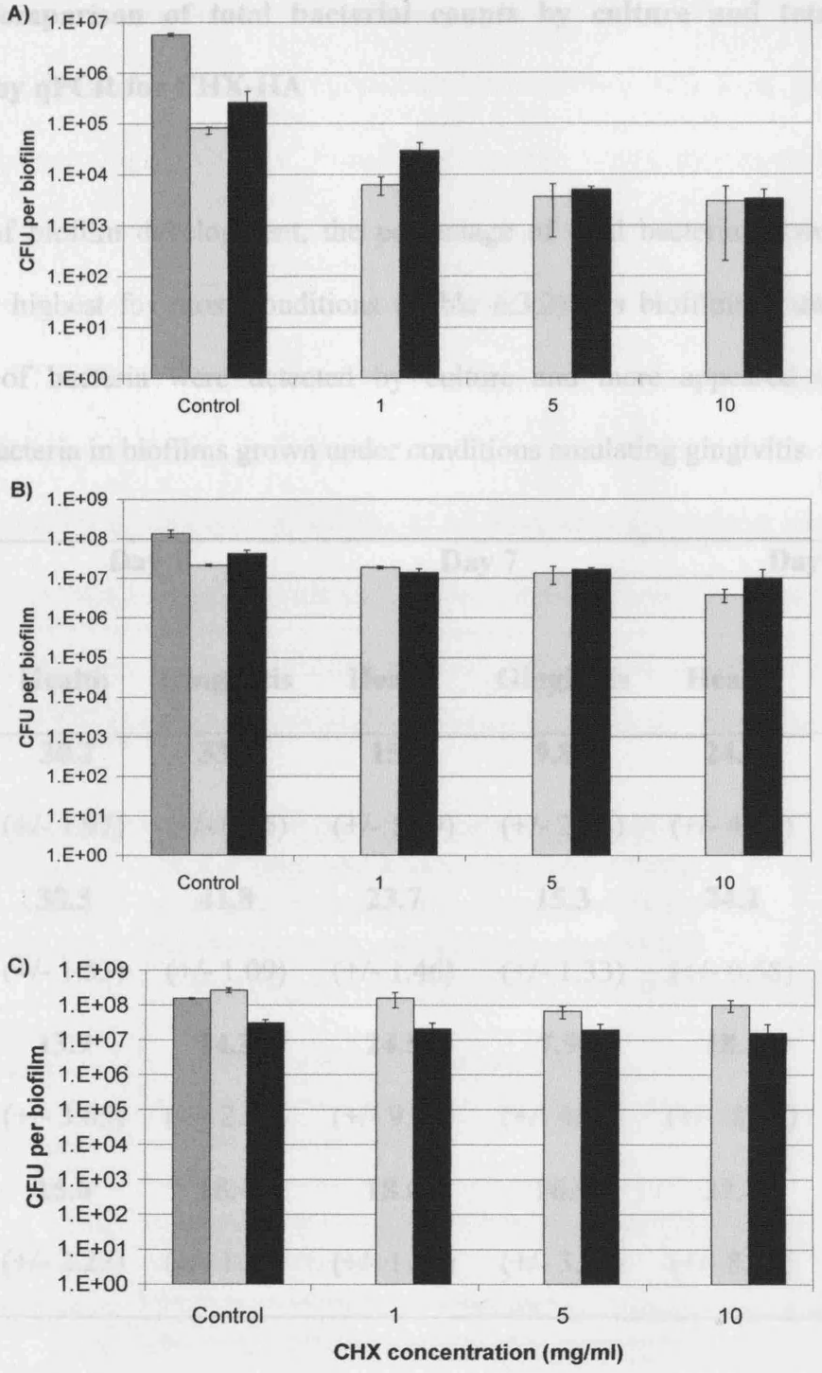

Figure 6.3.10: Total anaerobe counts on FAA under conditions emulating health or gingivitis for biofilms grown on CHX-HA. A) represents day 1, B) represents day 7, C) represents day 14 of biofilm development; ■ represents fermenter under conditions emulating health, ■ represents fermenter under conditions emulating gingivitis, ■ represents control from fermenter containing HA discs only. Error bars represent the standard deviations ($n = 6$).

6.3.3.2.2 Comparison of total bacterial counts by culture and total numbers calculated by qPCR for CHX-HA

On day 1 of biofilm development, the percentage of total bacteria accounted for by culture was highest for most conditions (Table 6.3.2). As biofilms matured a lower percentage of bacteria were detected by culture and there appeared to be fewer cultivable bacteria in biofilms grown under conditions emulating gingivitis.

CHX (mg/ml)	Day 1		Day 7		Day 14	
	Health	Gingivitis	Health	Gingivitis	Health	Gingivitis
1	**30.2**	**33.2**	**15.9**	**9.86**	**24.6**	**15.8**
	(+/- 1.97)	(+/- 0.76)	(+/- 1.29)	(+/- 2.41)	(+/- 4.79)	(+/- 4.63)
5	**38.5**	**41.8**	**23.7**	**15.3**	**24.2**	**18.2**
	(+/- 1.53)	(+/- 1.09)	(+/- 1.46)	(+/- 1.33)	(+/- 0.68)	(+/- 6.04)
10	**13.9**	**14.3**	**24.5**	**7.98**	**18.3**	**13.5**
	(+/- 3.65)	(+/- 2.31)	(+/- 9.06)	(+/- 4.81)	(+/- 8.52)	(+/- 4.30)
Control	**15.6**	**18.4**	**18.6**	**16.9**	**27.8**	**21.5**
	(+/- 2.22)	(+/- 1.68)	(+/- 1.22)	(+/- 3.56)	(+/- 8.08)	(+/-2.90)

Table 6.3.2: Total bacterial counts (CFUs) expressed as a percentage of the total bacterial numbers calculated by qPCR on CHX-HA. Data are means and standard deviations ($n = 6$).

6.3.3.2.3 Microbial composition of biofilms grown on CHX-HA

On day 1 of biofilm formation, microcosm communities had high proportions of *Streptococcus* spp (Figure 6.3.11). *Fusobacterium* spp. were only detected in very low numbers in the fermenter under conditions emulating gingivitis and on control HA discs from both conditions. *Prevotella* spp. and *A. naeslundii* were not detected, except for on the control HA discs. The total bacterial numbers were similar for all biofilms grown under conditions emulating health on day 1, regardless of CHX concentration. Increasing concentrations of CHX seemed to be more effective against biofilms grown under conditions emulating gingivitis as total bacterial numbers were less than controls under these conditions but this reduction was only statistically significant ($P < 0.01$) at the highest concentration of CHX.

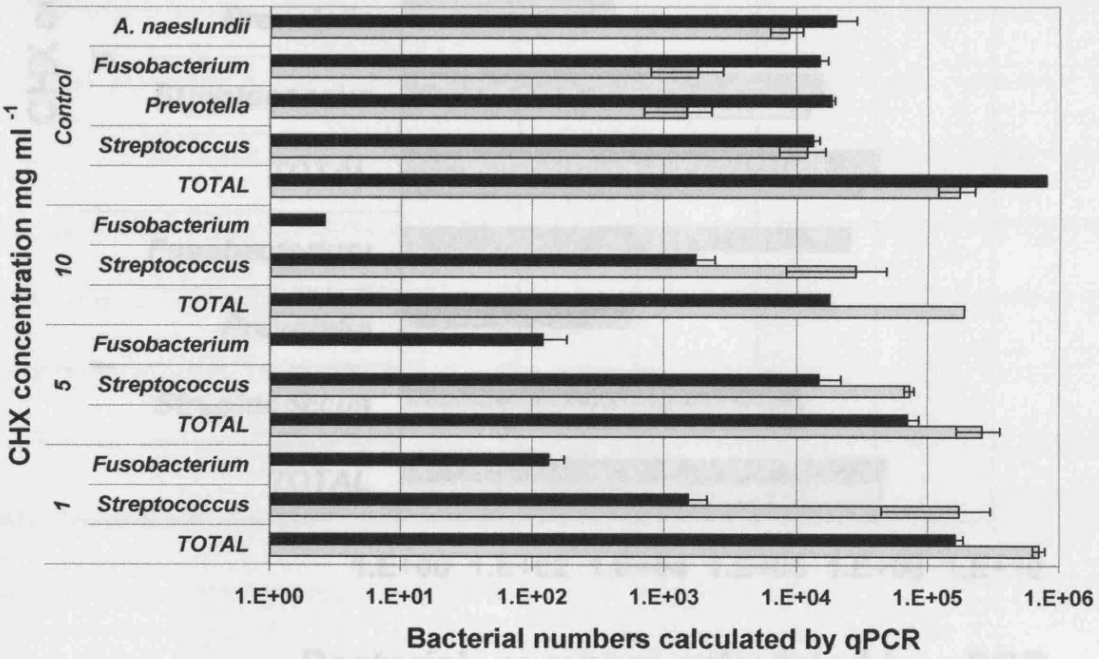

Figure 6.3.11: Bacterial numbers calculated by qPCR for CHX-HA discs on day 1 of biofilm development. ▪ represents fermenter under conditions emulating health, ■ represents fermenter under conditions emulating gingivitis. Error bars represent the standard deviations ($n = 6$)

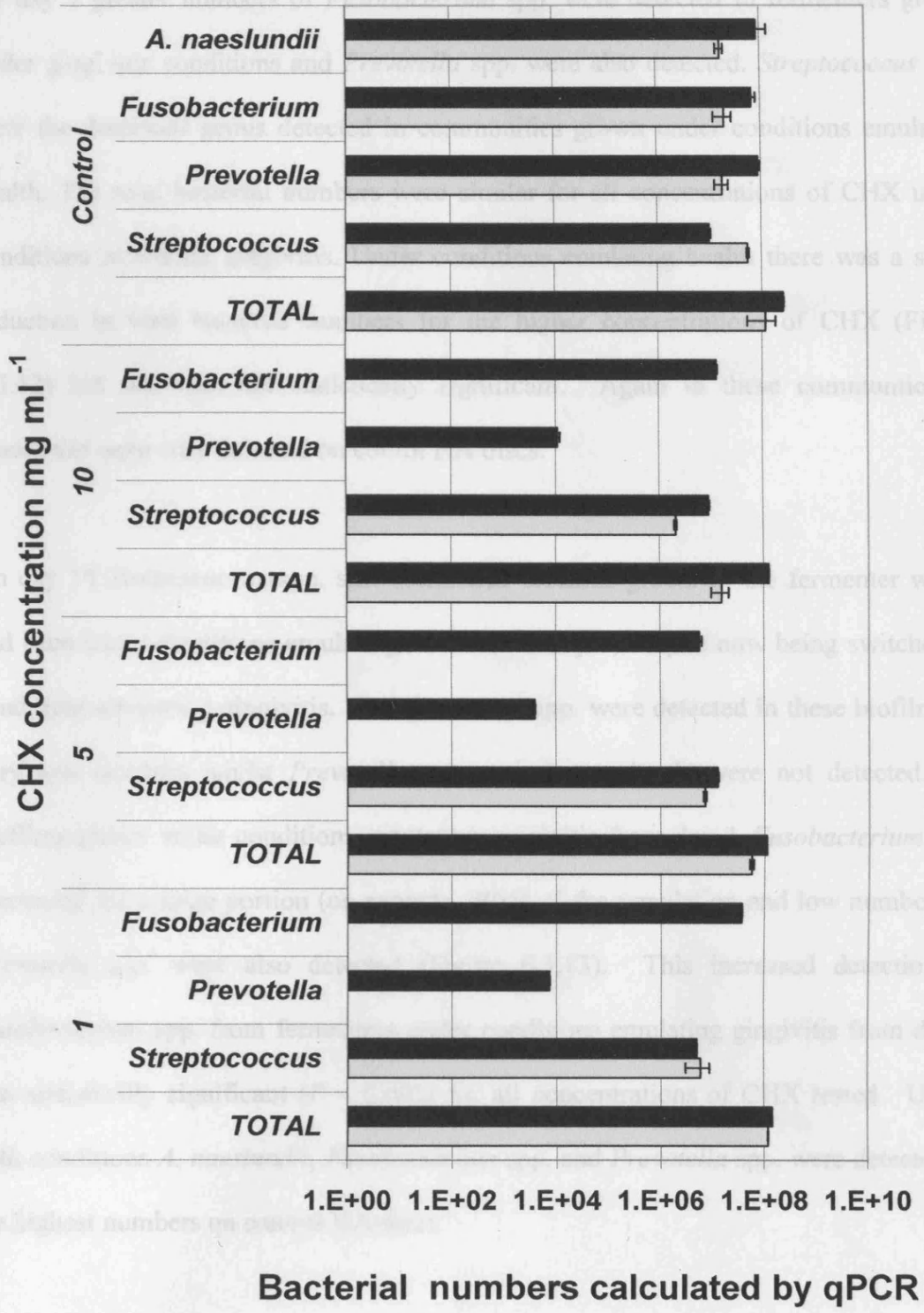

Figure 6.3.12: Bacterial numbers calculated by qPCR for CHX-HA discs on day 7 of biofilm development. ▪ represents fermenter under conditions emulating health, ■ represents fermenter under conditions emulating gingivitis. Error bars represent the standard deviations (n = 6).

By day 7 greater numbers of *Fusobacterium* spp. were detected in fermenters grown under gingivitis conditions and *Prevotella* spp. were also detected. *Streptococcus* spp. were the dominant genus detected in communities grown under conditions emulating health. The total bacterial numbers were similar for all concentrations of CHX under conditions emulating gingivitis. Under conditions emulating health there was a slight reduction in total bacterial numbers for the higher concentrations of CHX (Figure 6.3.12) but this was not statistically significant. Again in these communties *A. naeslundii* were only detected on conrol HA discs.

On day 14 *Streptococcus* spp. still dominated biofilms grown in the fermenter which had been under conditions emulating health until day 7, despite now being switched to conditions emulating gingivitis. *Fusobacterium* spp. were detected in these biofilms in very low numbers whilst *Prevotella* spp. and *A. naeslundii* were not detected. In biofilms grown under conditions emulating gingivitis from day 1 *Fusobacterium* spp. accounted for a large portion (on average, 30%) of the population and low numbers of *Prevotella* spp. were also detected (Figure 6.3.13). This increased detection of *Fusobacterium* spp. from fermenters under conditions emulating gingivitis from day 1 was statistically significant ($P < 0.001$) for all concentrations of CHX tested. Under both conditions *A. naeslundii*, *Fusobacterium* spp. and *Prevotella* spp. were detected in the highest numbers on control HA discs.

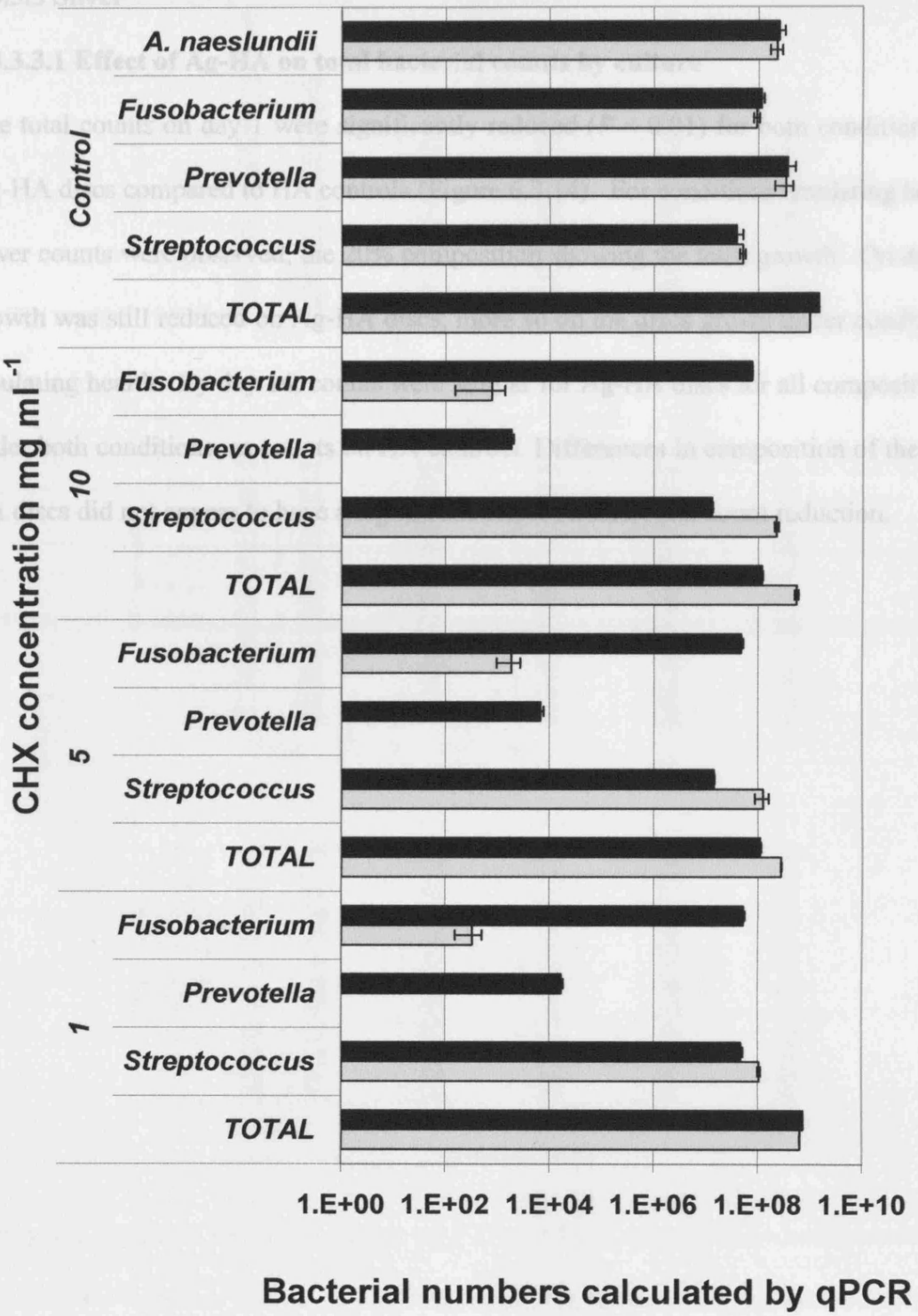

Figure 6.3.13: Bacterial numbers calculated by qPCR for CHX-HA discs on day 14 of biofilm development. ■ represents fermenter under conditions emulating health, then switched to conditions emulating gingivitis on day 7, ■ represents fermenter under conditions emulating gingivitis. Error bars represent the standard deviations ($n = 6$).

6.3.3.3 Silver

6.3.3.3.1 Effect of Ag-HA on total bacterial counts by culture

The total counts on day 1 were significantly reduced ($P < 0.01$) for both conditions on Ag-HA discs compared to HA controls (Figure 6.3.14). For conditions emulating health lower counts were observed, the 20% composition showing the least growth. On day 7, growth was still reduced on Ag-HA discs, more so on the discs grown under conditions emulating health. By day 14, counts were similar for Ag-HA discs for all compositions, under both conditions, as counts on HA controls. Differences in composition of the Ag-HA discs did not appear to have a significant effect on microbial count reduction.

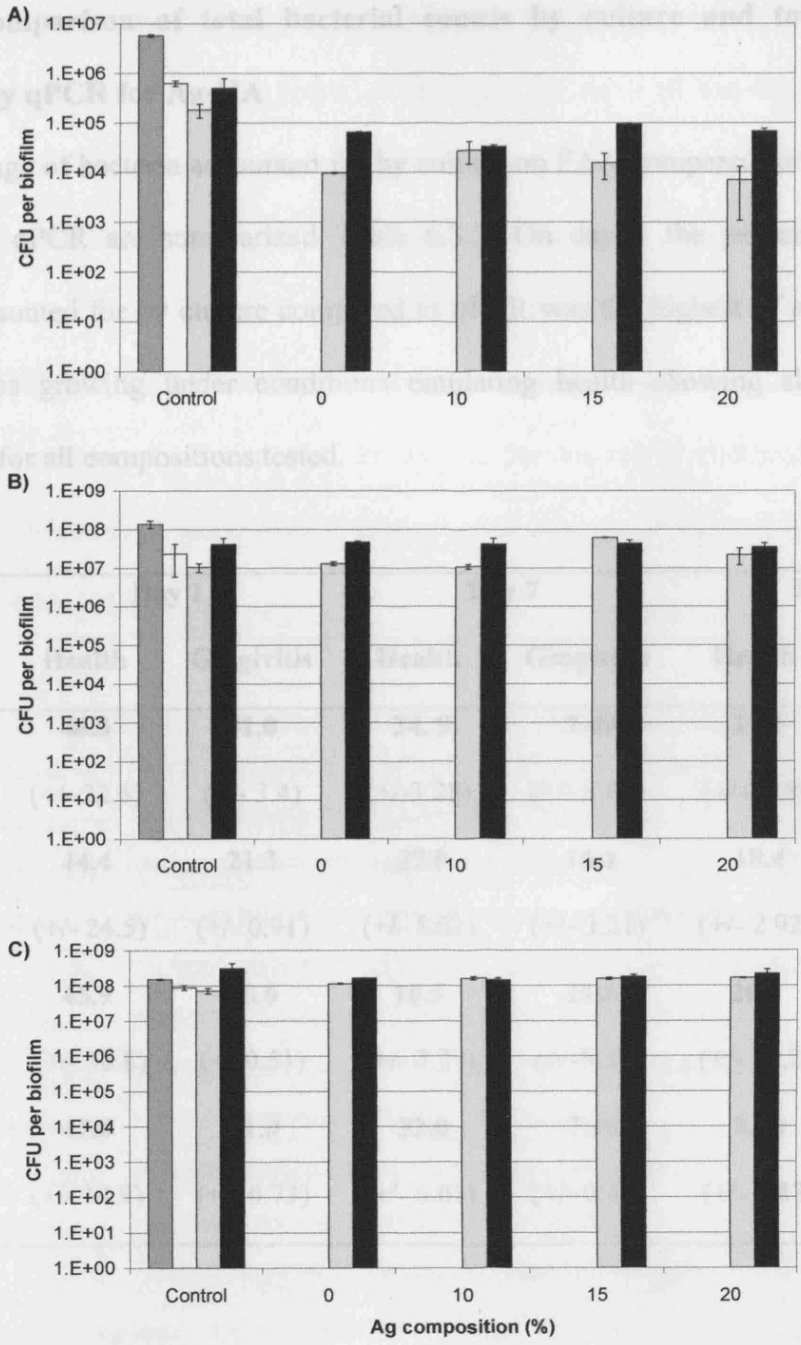

Figure 6.3.14: Total anaerobe counts on FAA under conditions emulating health or gingivitis for biofilms grown on Ag-HA. A) represents day 1, B) represents day 7, C) represents day 14 of biofilm development; ■ represents fermenter under conditions emulating health, ■ represents fermenter under conditions emulating gingivitis, □ represents HA control, ■ represents control from fermenter containing HA discs only. Error bars represent the standard deviations ($n = 6$).

6.3.3.3.2 Comparison of total bacterial counts by culture and total numbers calculated by qPCR for Ag-HA

The percentage of bacteria accounted for by culture on FAA compared to total bacterial numbers by qPCR are summarized Table 6.3.3. On day 1 the percentage of total bacteria accounted for by culture compared to qPCR was the highest of all days tested with biofilms growing under conditions emulating health showing slightly higher percentages for all compositions tested.

Ag	Day 1		Day 7		Day 14	
composition	**Health**	**Gingivitis**	**Health**	**Gingivitis**	**Health**	**Gingivitis**
10 %	**46.3**	**41.0**	**24. 9**	**7.49**	**17.3**	**6.64**
	(+/- 22.6)	(+/- 3.4)	(+/-3.28)	(+/- 3.09)	(+/-2.18)	(+/- 1.49)
15 %	**44.4**	**21.3**	**27.0**	**16.1**	**18.4**	**15.5**
	(+/- 24.5)	(+/- 0.91)	(+/- 5.62)	(+/- 3.21)	(+/- 2.92)	(+/- 1.97)
20 %	**45.9**	**38.6**	**16.5**	**21.2**	**26.7**	**15.9**
	(+/- 38.8)	(+/-0.51)	(+/- 7.77)	(+/-5.38)	(+/- 11.2)	(+/-0.72)
Control	**47.0**	**31.0**	**32.0**	**7.99**	**9.90**	**7.93**
	(+/- 14.9)	(+/- 0.73)	(+/- 6.01)	(+/- 0.45)	(+/- 3.87)	(+/- 0.20)

Table 6.3.3: Total bacterial counts (CFUs) expressed as a percentage of the total bacterial numbers calculated by qPCR on Ag-HA. Data are means and standard deviations ($n = 6$).

6.3.3.3.3 Microbial composition of biofilms grown on Ag-HA

On day 1 *Streptococcus* spp. appeared to account for most of the bacteria detected (Figure 6.3.15). Total bacterial numbers for biofilms grown under conditions emulating gingivitis were significantly higher ($P < 0.05$) than biofilms grown under conditions emulating health for the 20% compositions and controls. For biofilms grown under conditions emulating health there appeared to be no reduction in bacterial numbers on Ag-HA discs compared to controls. However, the microbial composition of these biofilms was altered as *A. naeslundii*, *Fusobacterium* spp. and *Prevotella* spp. were only detected on control HA discs.

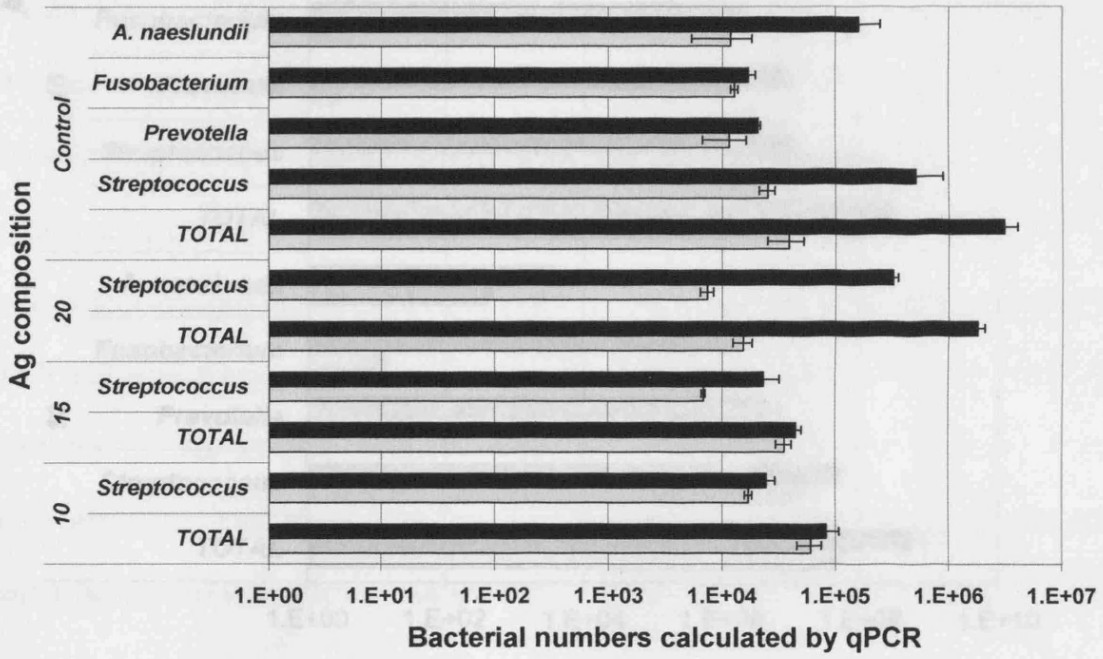

Figure 6.3.15: Bacterial numbers calculated by qPCR for Ag-HA discs on day 1 of biofilm development. ■ represents fermenter under conditions emulating health, ■ represents fermenter under conditions emulating gingivitis. Error bars represent the standard deviations ($n = 6$).

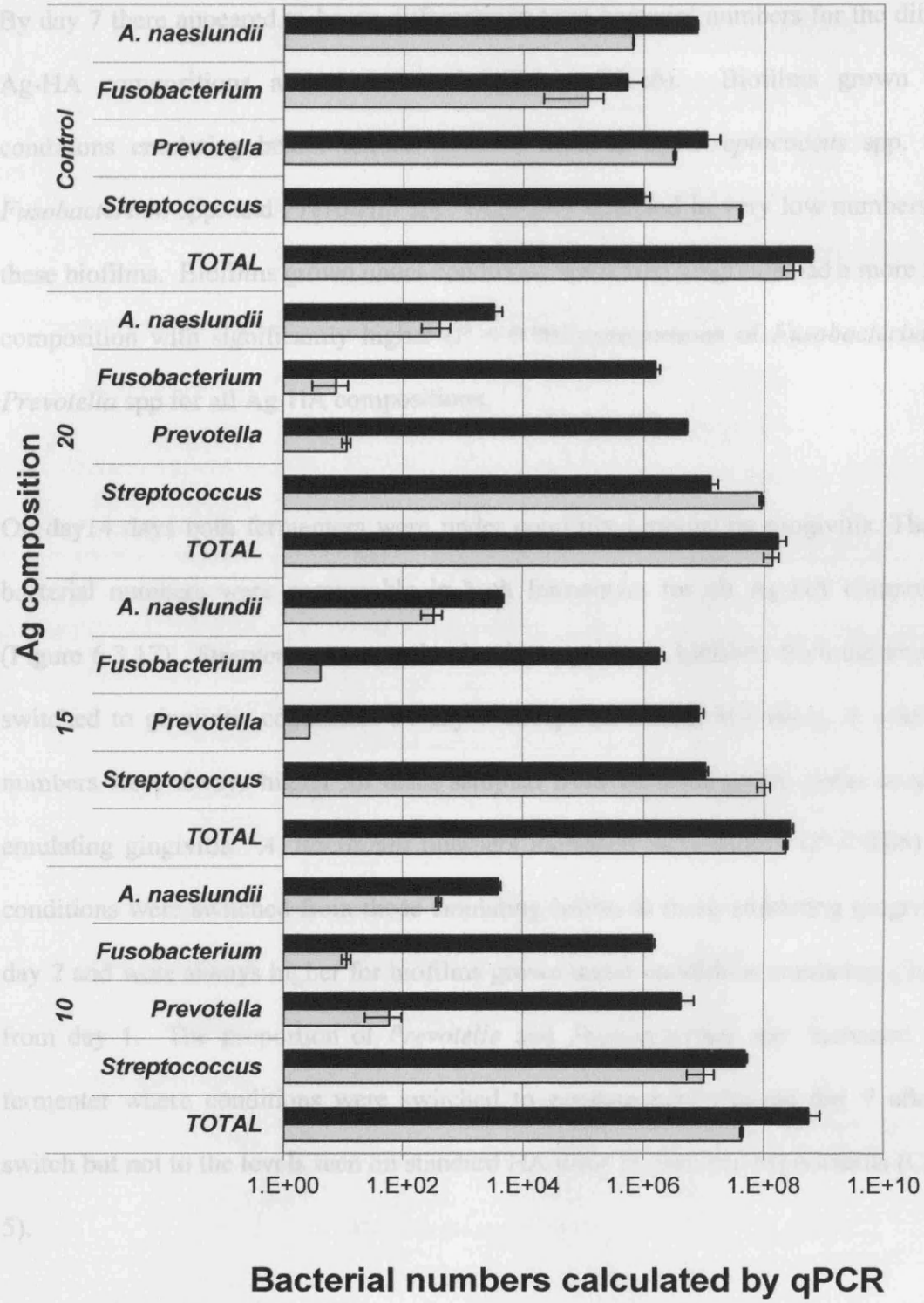

Figure 6.3.16: Bacterial numbers calculated by qPCR for Ag-HA discs on day 7 of biofilm development. ■ represents fermenter under conditions emulating health, ■ represents fermenter under conditions emulating gingivitis. Error bars represent the standard deviations (n = 6).

By day 7 there appeared to be no difference in total bacterial numbers for the different Ag-HA compositions and the controls (Figure 6.3.16). Biofilms grown under conditions emulating health tended to be dominated by *Streptococcus* spp. whilst *Fusobacterium* spp. and *Prevotella* spp. were only detected in very low numbers from these biofilms. Biofilms grown under conditions emulating gingivitis had a more mixed composition with significantly higher ($P < 0.001$) proportions of *Fusobacterium* and *Prevotella* spp for all Ag-HA compositions.

On day14 days both fermenters were under conditions emulating gingivitis. The total bacterial numbers were comparable in both fermenters for all Ag-HA compositions (Figure 6.3.17). *Streptococcus* was the dominant genus in biofilms from the fermenter switched to gingivitis conditions on day 7 except on control HA discs. *A. naeslundii* numbers were always higher for discs sampled from biofilms grown under conditions emulating gingivitis. *A. naeslundii* numbers increased significantly ($P < 0.05$) when conditions were switched from those emulating health to those emulating gingivitis on day 7 and were always higher for biofilms grown under conditions emulating gingivitis from day 1. The proportion of *Prevotella* and *Fusobacterium* spp. increased in the fermenter where conditions were switched to emulate gingivitis on day 7 after this switch but not to the levels seen on standard HA discs in previous experiments (Chapter 5).

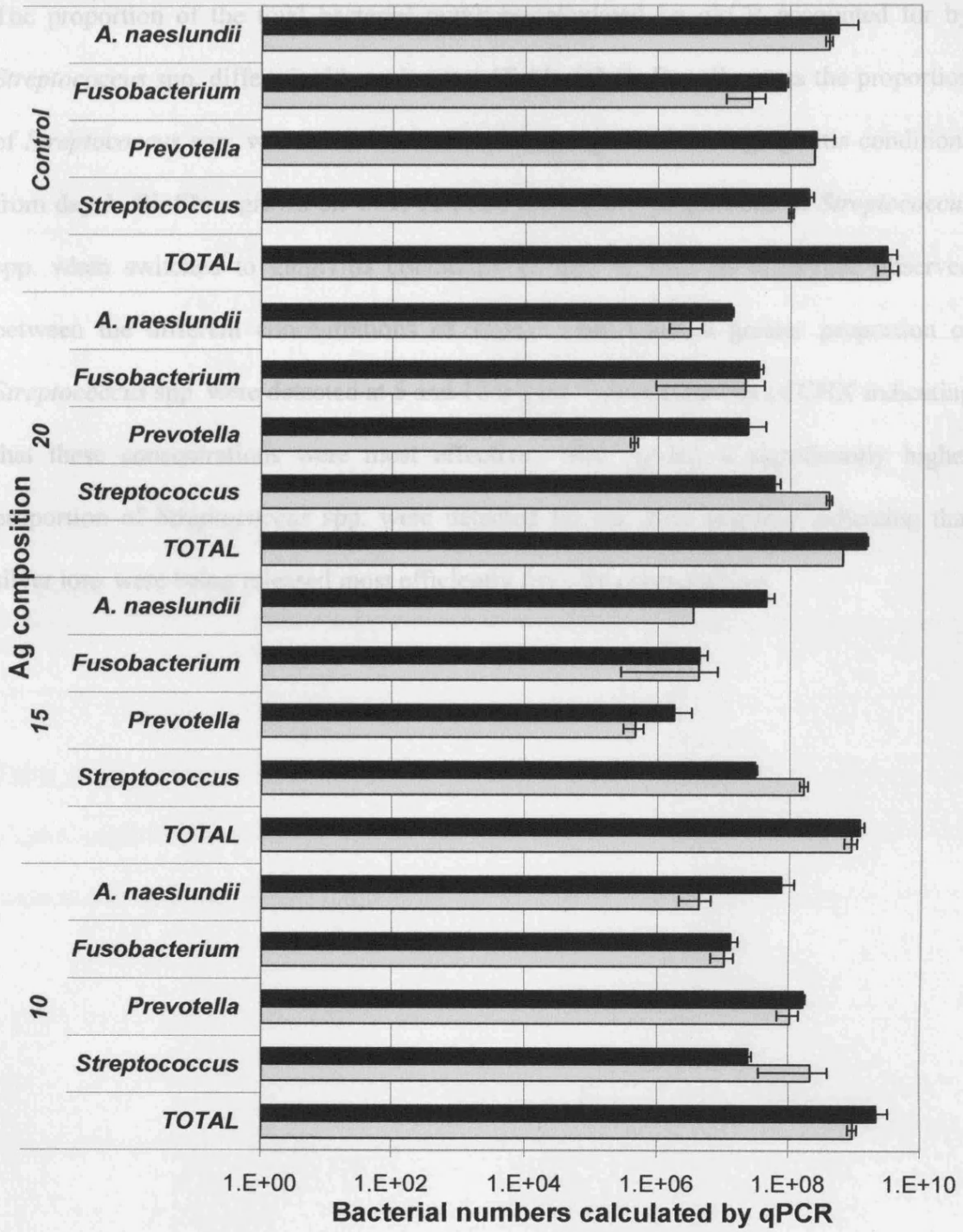

Figure 6.3.17: Bacterial numbers calculated by qPCR for Ag-HA discs on day 14 of biofilm development. ■ represents fermenter under conditions emulating health, then switched to conditions emulating gingivitis on day 7, ■ represents fermenter under conditions emulating gingivitis. Error bars represent the standard deviations ($n = 6$).

The proportion of the total bacterial numbers calculated by qPCR accounted for by *Streptococcus* spp. differed with each agent (Table 6.3.4). For all agents the proportion of *Streptococcus* spp. was much lower for biofilms grown under gingivitis conditions from day 1. Biofilms grown on TCH-HA had the highest proportions of *Streptococcus* spp. when switched to gingivitis conditions on day 7, with no difference observed between the different concentrations of TCH. For CHX a greater proportion of *Streptococcus* spp. were detected at 5 and 10 mg ml $^{-1}$ concentrations of CHX indicating that these concentrations were most effective. For Ag-HA a significantly higher proportion of *Streptococcus* spp. were detected for the 20% possibly indicating that silver ions were being released most efficiently from this composition.

	Agent adsorbed / incorporated into HA					
	TCH		CHX		Ag	
Agent concentration	Health to Gingivitis	Gingivitis	Health to Gingivitis	Gingivitis	Health to Gingivitis	Gingivitis
A	68.6	20.5	16.5	6.46	22.9	1.11
	(+/- 6.94)	(+/- 2.88)	(+/-1.05)	(+/- 0.55)	(+/- 1.92)	(+/- 0.14)
B	63.7	39.0	44.9	13.2	19.0	2.60
	(+/- 25.5)	(+/- 13.1)	(+/- 13.3)	(+/- 0.36)	(+/- 2.69)	(+/- 0.18)
C	68.0	21.6	41.2	11.1	62.5	41.9
	(+/- 8.02)	(+/- 2.13)	(+/- 3.96)	(+/- 0.59)	(+/- 6.40)	(+/- 0.96)
Control	6.73	6.28	5.25	2.66	3.22	6.48
	(+/- 0.46)	(+/- 0.16)	(+/- 0.68)	(+/- 0.88)	(+/- 0.03)	(+/- 0.12)

Table 6.3.4: Proportion of *Streptococcus* spp. on day 14 of biofilm development. (Agent concentrations A, B, and C are 1, 5 and 10 mg ml $^{-1}$ of TCH or CHX respectively. For Ag-HA A, B and C are 10, 15 and 20 % glass respectively).

6. 4 Discussion

6.4.1 Effectiveness of different therapeutic agents against plaque accumulation

Biofilms grown under conditions emulating gingivitis generally had higher total bacterial numbers, most probably due to the increased availability of nutrients under these conditions. For all of the agents tested the greatest effect on bacterial numbers determined by culture and qPCR was on day 1 of biofilm development. All of the agents were being released from the surface of the HA discs and thus during initial attachment of cells would be acting on individual cells rather than biofilms thereby making them more effective. The main influence of an antibacterial agent being released from the HA surface was initial inhibition of biofilm formation as planktonic initial colonizers would be killed by the action of the antibacterial agent making the rate at which bacteria attach to the enamel surface slower. Chlorhexidine binds to salivary pellicle proteins (Reed *et al,* 1981) and is thus likely to have a prolonged effect against initial colonizers such as adherent streptococci. Tetracycline strongly binds to hydroxyapatite (Misra, 1991) and has been shown to inhibit *in vitro* plaque formation by pure cultures of oral bacteria (Baker *et al,* 1983). Biofilm formation by *S. sanguinis* on phosphate-based glasses containing silver ions has also shown to be significantly reduced for the first 48 hours of biofilm development (Mulligan *et al,* 2003). Once biofilms had become established at day 7 all of the agents were less effective, with only TCH showing significant reductions at higher concentrations when compared to controls. By day 14 there appeared to be no difference in bacterial numbers on HA-treated discs or controls. This could be due to all of the respective agents having been released by this point or due to failure of agents to penetrate beyond layers of the biofilm closest to the HA surface, which have been shown to contain higher proportions of nonviable bacteria by viability staining techniques (Hope *et al,* 2002).

From the adsorption and release data TCH was released at higher concentrations than CHX as the amount of CHX being released was too low and out of the range of calibration. Verification that CHX was having some antibacterial effect was obtained by incubation with planktonic *S. sanguinis* against which they did show a significant reduction in numbers after 24 hours. The total amount of TCH adsorbed into HA was higher than the amount of CHX. By comparison of the total bacterial numbers it was apparent that TCH showed prolonged sustained release and bacterial numbers were still reduced after 7 days of biofilm development. After 7 days the amount of TCH being released from HA had plateaued indicating that after this point no more TCH was being released. Indeed after this point no reduction was observed in the total bacterial numbers. In contrast, silver ions were incorporated into HA discs rather than being adsorbed into HA (as with TCH and CHX) and so would be expected to be released at a slower rate. The release of this agent was not quantified but as the microbial communities developing on these were different to those growing on HA in terms of overall numbers (at least initially) it can be assumed that silver ions were being released and were having an antibacterial effect, although not as great as chlorhexidine and tetracycline.

6.4.2 Influence of therapeutic agents on microbial composition of oral biofilms

The influence of all the agents used was tested using qPCR rather than traditional culture techniques which had already been used in the previous chapters to characterize these communities. The use of these techniques was much less time consuming than culture though more costly using the qPCR reagents. The percentage of the total bacteria accounted for by culture was less when experiments were carried out under conditions emulating gingivitis, even on day 1 of biofilm development. This is most

likely due to these communities containing higher proportions of species which are more difficult to culture. From qPCR it was ascertained that these communities harboured higher numbers of *Prevotella* and *Fusobacterium* spp. which were rarely isolated using the culture techniques described in previous chapters. As biofilms developed the proportion of bacteria accounted for by culture decreased. If this was simply due to these communities containing higher proportions of nonviable cells it would be expected that control HA discs should have the highest proportion of cultivable (or 'viable') bacteria. This was not the case, as the control HA discs often had the lowest proportion of cultivable bacteria, indicating that these communities had the lowest proportions of bacteria which could be detected by the culture techniques used and this difference in proportions was reflective of differences in microbial composition. It is thought that there is a rapid turnover of dead cells in the oral cavity as significant levels of proteolytic activity have been detected in the oral microbial communities (Soder, 1972; Wei *et al*, 1999) and oral bacteria have been shown to have nuclease activity which would degrade DNA from broken down dead cells.

6.4.2.1 Influence of TCH-HA

TCH appeared to have the broadest spectrum of action of all the agents tested as only *Streptococcus* spp. were detected by qPCR. *Prevotella* spp., *Fusobacterium* spp. and *A. naeslundii* were not detected, even after 14 days under conditions emulating gingivitis. On control discs containing no agent these organisms were detected and so the elimination of these organisms from the biofilm communtites can be directly attributed to the presence of TCH. As these organisms are not usually present in high numbers until some days after initial biofilm development if their numbers are significantly reduced initially by TCH then they may never become established, even when

conditions that favour their growth develop. TCH has been shown to significantly reduce *A. naeslundii* numbers in experimental gingivitis studies on hamsters (Nomura, 1989) and significantly reduced numbers in periodontitis patients where tetracycline releasing fibres have been used as treatment (Sakellari *et al*, 2003). Local delivery of tetracycline as therapy for peri-implantitis was shown to have a positive effect on clinical and microbiological parameters (Mombelli *et al,* 2001) with significant reductions in *Pr. intermedia*, *Pr. nigrescens*, *Fusobacterium* spp., *B. forsythus*, and *C. rectus*. The adsorption of tetracycline to saliva-coated enamel has been shown to prevent the formation of biofilms of pure cultures of *A. viscosus* and *A. naeslundii* (Baker *et al*, 1983). Dental materials containing tetracycline have also been shown to inhibit growth of *A. israelii* and *A. naeslundii* (Schaeken & De Haan, 1989). The influence of pulsing established oral biofilms developed in the CDFF with TCH at various time intervals (Ready *et al,* 2002) resulted in reduced bacterial numbers immediately after application followed by a recovery in total counts but not to levels seen before pulsing. *Actinomyces* spp. were reduced after pulsing (by 96%) but the proportion of the total community that they accounted for remained the same, while the proportion of *Streptococcus* spp. was significantly reduced with communities becoming dominated by *Lactobacillus* spp. which are implicated in the development of dental caries due to their production of lactic acid. A community dominated by *Streptococcus* spp. is more conducive to the maintenance of oral health and thus the action of TCH against developing biofilms is more likely to achieve this. As *Streptococcus* spp. are the dominant organisms in developing plaque even if numbers are significantly reduced initially by TCH action this may only lower the rate of initial biofilm formation by these species after which numbers can still recover as the environmental conditions are not yet suitable for other species to become the dominant organisms. One drawback to the

prolonged use of tetracycline is the enrichment for bacteria resistant to tetracycline and other agents observed in microcosm communities (Ready *et al,* 2002). Short-term use in high risk patients such as those already suffering from more severe forms of periodontal disease or recent dental surgery would be the most suitable application of tetracycline in the oral cavity rather than continuous exposure as the microbial composition of dental plaque was significantly altered by this agent.

6.4.2.2 Influence of CHX-HA

The microbial communities which developed on CHX-HA contained *Fusobacterium* and *Prevotella* spp. under conditions emulating gingivitis, but in reduced numbers when compared to control HA discs. *Fusobacterium* spp. were detected in higher numbers than *Prevotella* spp. indicating that they were less susceptible to CHX. *A. naeslundii* were not detected at all in these communities, except for on control HA discs and therefore, as with TCH, the elimination of this organism from the community can be directly attributed to the application of CHX. Chlorhexidine is an effective antimicrobial agent against planktonic periodontal pathogens such as *Pr. intermedia*, *P. gingivalis* (Vianna *et al,* 2004) and *F. nucleatum* (Sena *et al,* 2006; Carson *et al*, 2005). Used against *Pr. intermedia*, *Pr. denticola* and *Pr. melaninogenica*, *Pr. denticola* was found to be most susceptible (Do Amorim *et al,* 2004). In a study by McBain *et al* (2003b), *A. naeslundii*, *V. dispar*, *Pr. nigrescens* and streptococci were found to be very susceptible to topical application of CHX in microcosm communities, *A. naeslundii* not being detected at all from these communities (Mc Bain *et al,* 2003b). The effect of repeated pulsing with chlorhexidine on oral biofilms developed in the CDFF (Pratten *et al,* 1998a) was that populations gradually became resistant so that little or no effect was seen, even immediately after pulsing. *A. naeslundii* which had been present before

pulsing was lost from these communities. Pre-treating of the enamel discs on which biofilms would form with chlorhexidine resulted in *Actinomyces* spp. not being detected at all which is similar to the observations of this study. The use of chlorhexidine varnishes on enamel *in vivo* has been shown to suppress *A. naeslundii* and *A. viscosus* for up to two weeks after application (Schaeken & de Haan, 1989). Interestingly there was also no suppression in the total cultivable flora or *S. sanguinis* with the use of chlorhexidine varnish. Although proven to be an effective agent for plaque reduction and the prevention of gingivitis (Loe & Schiott, 1970; Jenkins *et al,* 1994) chlorhexidine is not an ideal candidate for prolonged use in the oral cavity due to the resulting staining of teeth (Addy *et al,* 1982, 1989) and therefore, as with tetracycline, it is most suitable for short term application.

6.4.2.3 Influence of Ag-HA

The microbial communities that developed on Ag-HA were the most varied of the three agents tested. These communities were again initially dominated by *Streptococcus* spp. but *Fusobacterium* spp., *Prevotella* spp. and *A. naeslundii* all accounted for significant portions of the total community detected by qPCR in mature biofilms, especially under conditions emulating gingivitis. However, when compared to the total numbers detected for these species by qPCR, under conditions emulating gingivitis on HA discs (Chapter 5), the numbers of *Prevotella* spp. were significantly reduced whilst *Fusobacterium* spp. and *A. naeslundii* numbers were only slightly reduced indicating that they were less susceptible. Prolonged use in the oral cavity via controlled release local delivery devices be may more appropriate with this agent as the microbial composition of microcosm communities was not as significantly altered, with proportions of species associated with periodontal disease being reduced with increased

proportions of streptococci, both changes which would be beneficial in the prevention of periodontal diseases. Silver ions have been shown to be more effective against periodontal pathogens than oral streptococci and retain this killing activity in human serum (Spacciapoli *et al,* 2001). Silver ions are effective agents for reducing infections in burn and wound patients and are used in face creams and washing of foodstuffs without adverse effects on humans (Silver, 2003). Silver ions have been shown to reduce *S. aureus* biofilm formation (Akiyama *et al,* 1998) and release from phosphate-based glasses has been shown to reduce *S. sanguinis* biofilm formation, at least in the first 48 hours. Sustained release of silver from periodontal wafers has been shown to reduce bacterial numbers for at least 21 days (Bromberg *et al,* 2000) with minimal side effects on hard and soft tissues.

6.4.3 Conclusions

The results of this chapter have added to the understanding of the influences of different therapeutic agents on the microbial composition of oral biofilm communities in an attempt to assess their efficacy against the accumulation of dental plaque. Although total bacterial numbers did not seem to be affected after initial biofilm formation the microbial communities which developed in the presence of these different agents differed from those developed in CDFF experiments where no agent was used. With all agents a greater portion of the total bacterial community was accounted for by *Streptococcus* spp., even under conditions emulating gingivitis compared to controls, indicating that a microbial composition associated with health was encouraged with the use of these agents at their most effective concentrations. The application of incorporation of antibacterial agents into dental materials is for local delivery and sustained release of therapeutic agents into the oral cavity, for example, for use with

dental implants to reduce the risk of rejection due to peri-implantitis, which in turn would have a significant impact on dental costs.

CHAPTER 7

Final Discussion

Periodontal diseases are some of the most prevalent diseases affecting humans and impact on most individuals at some point in their lives. Several factors such as health, age, socioeconomic status, dental care usage, smoking and the use of antimicrobials are influential in the development of these microbial periodontal diseases (Douglas *et al*, 1983). However, understanding the complex microbial communities that are associated with oral health and disease is crucial in developing methods to prevent their inception and progression.

The first part of this study was concerned with establishing a reproducible *in vitro* model for determining microbial population changes that occur during gingivitis development. As oral microbial communities are so complex the initial studies focused on two key genera, *Streptococcus* and *Actinomyces* spp., that would be indicative of the changes in the microbial community associated with gingivitis. These genera were chosen because of their association with oral health and gingivitis (Moore *et al*, 1982; Moore *et al*, 1987). Furthermore, they are present in the dental plaque of most individuals, can be isolated using traditional culture techniques and are relatively easy to compare with other oral species. *Actinomyces* spp. are by no means the only species associated with gingivitis but changes in these species proportions are an indicator of changes occurring in the oral microbial community associated with gingivitis (Loesche & Syed 1978; Moore *et al*,1984).

According to the ecological plaque hypothesis (Marsh, 1991b) the inflammatory response instigated by plaque accumulation leads to a higher flow rate of GCF which is one of the key environmental factors in the development of gingivitis. The results of this study follow this hypothesis as the environmental parameters which were most

influential on the microbial composition of the biofilms grown in the model were oxygen availability and the presence of an additional nutrient source, artificial GCF. Whilst changes in these environmental factors could be emulated in the CDFF model more host derived materials could be incorporated into the model to make it more representative of the complexity of the oral cavity environment. Microbial interactions with host molecules, and programmed responses to host environmental stimuli, are critical for colonization and initiation of pathogenesis (Heddle *et al*, 2003). This was attempted with collagen coating of the hydroxyapatite substratum as collagen has been shown to interact with oral streptococci (Switalski *et al*, 1993) playing a role in their attachment to oral surfaces. However, as this coating had no appreciable effect on the biofilms that developed this approach was not pursued. As the salivary pellicle is formed almost instantaneously on exposure of enamel to the oral environment (Hannig, 1999) and this pellicle formation reaches an equilibrium of adsorption and de-sorption within 2 hours (Lendenmann *et al*, 2000) it is highly likely that the interaction of oral bacteria with components of the salivary pellicle is the most influential on initial bacterial attachment and biofilm formation (Scannapieco, 1994). For example, the bacterial proteolysis of salivary proline rich proteins releases a host peptide which is thought to induce a biofilm associated phenotype in oral streptococci (Drobni *et al*, 2006) and there are countless other examples of oral bacterial interactions with salivary components (Jenkinson *et al*, 1993; Blehert *et al*, 2003; Edwards *et al*, 2007). Therefore the influence of other host molecules on biofilm formation was not investigated in this study.

It was important to be able to characterize the microbial communities present in the model to ensure that it was representative of the population *in vivo*. Defining the oral

microbiota is a task which has evolved with the development of more representative methods of microbial community assessment. Techniques which have been applied to other diverse microbial ecosystems have been applied to dental plaque to gain a better knowledge of the community as a whole. For example, with the advance of molecular techniques such as sequencing of the 16S rRNA gene it is possible to get more accurate identification of species as well as defining new taxa, species and genera. Furthermore, the use of culture-independent techniques such as PCR and cloning have allowed for the identification of uncultivable species and facilitated the identification of species which are more difficult to identify with more traditional techniques. The microcosm model used in this study has previously been characterized using traditional culture techniques, amplification and cloning of the 16S rRNA gene (Pratten *et al*, 2003a) and DGGE (McBain *et al*, 2003b).

Amplification and cloning of the 16S rRNA gene is important in identifying novel uncultivable species which would not be represented by using culture techniques alone and may also facilitate the identification of species with more complex growth requirements. The main value of this technique is assessment of species richness and identification of novel species. Identification of novel species present in this model was not an aim of this study and thus this technique was not applied. DGGE has been used in soil communities to assess microbial diversity (Borneman *et al*, 1996) and to produce reproducible, distinct community fingerprints (Nakatsu *et al*, 2000). DGGE was considered as an assessment technique for the biofilm communities produced in this study, as it has the potential to create a community fingerprint for communities associated with health and disease. However, previous attempts to use DGGE for this purpose for microcosm communities developed in the CDFF have not been successful

as only a few distinct bands were observed per sample (McBain *et al*, 2003b). Also, the co-migration of bands from different species to the same position in the gel could mask the true richness of the community being examined (Gafan & Spratt, 2005), thus this technique was not used as a method to characterize the oral communities present in this model. Assessment of complex microbial communities has now entered the metagenomic era (Riesenfeld *et al*, 2004; Xu, 2006; Booijink *et al*, 2007) where by the genetic complement of the entire community can be assessed. Metagenomics have been applied to the complex communities of the human gastrointestinal tract, where large scale shifts in the commensal populations have been linked with the development of diseases associated with that habitat (Frank & Pace, 2008). Analysis of the oral metagenome is currently underway.

Monitoring changes in key species and genera using qPCR was thought to the best method of defining the communities associated with health and disease. The results showed that *Prevotella* spp., *A. naeslundii* and *P. gingivalis* all increased under conditions emulating gingivitis, whilst *Streptococcus* spp. decreased. The value of using qPCR as opposed to traditional culture techniques was the identification and quantification of species that were not detected by culture. This was particularly true for *Prevotella* spp. and *Fusobacterium* spp. which were rarely, if at all, isolated by culture techniques. The identification of species by culture and 16S rRNA sequencing of isolates is extremely time-consuming and many species can be missed using this technique as their presence can be masked by the occurrence of species which are more suited to growth on culture media. This dilution effect is important as some species that are implicated in disease may only be present as a small proportion of the community (Pratten *et al*, 2003). Ideally, more species could have been quantified using qPCR,

particularly species such as *Capnocytophaga* spp., *Eubacterium* spp., *Treponema* spp., *A. actinomycetemcomitans* and *T. forsythia* which have all been linked with periodontal diseases. This was not possible due to time and financial constraints due to the number of qPCR experiments that would need to be performed. Multiplex qPCR, for the detection of several oral species in the same reaction may aid in reducing these numbers and could be a direction for future work. This technique uses probes labelled with different coloured fluorophores (Tyagi *et al*, 1998; Wittwer *et al*, 2001), using a common quencher molecule. Using this technique it is possible to distinguish sequences with a single nucleotide difference in a single reaction (Marras *et al*, 1999). These types of probes also have thermodynamic properties that enhance their specificity (Bonnet *et al*, 1999). This technique has been applied to the simultaneous detection and quantification of different pathogens in clinical samples (Welti *et al*, 2003; Wada *et al*, 2007). Also, more recently DNA microarray technologies have been applied to complex microbial communities such as the gastrointestinal tract (Wang *et al*, 2004). Using this technique a large number of oligonucleotide probes with different characteristics can be applied in parallel and hence it is possible to define the presence of a species and also quantify them as the amount of fluorescence is proportional to bacterial numbers.

The ecological niches that develop within a biofilm can be occupied by a range of organisms with similar growth requirements and oral species have been grouped together according to these requirements (Socransky *et al*, 1998). During initial plaque development species with less complex growth requirements and the ability to adhere to the tooth surface dominate. Aerobic *Neisseria* spp. and aerotolerant *Streptococcus* and *Gemella* spp. form this proposed core group, which might provide a favourable biofilm

niche for subsequent or concomitant colonization of facultative and obligate anaerobes (Diaz *et al*, 2006). As plaque matures the variety of microenvironments present within a biofilm tend to increase and thus the diversity of species increases. Thus, the species which are present will also have more complex growth requirements, utilize different growth sources and ultimately produce different metabolic end products. Future studies on dental plaque development may focus on these interactions rather than the study of individual species as these may be more indicative of the stage of disease and will provide more information on the interactions taking place in microbial communities. The microbial complexity of dental plaque is linked to the variety of metabolic processes taking place, involving various methods of microbial communication and interaction. Metabolomics would provide key information on what metabolic processes are taking place within the microbial community. Concentration changes of specific groups of metabolites may reflect community responses to environmental changes. Thus, the study of metabolites is a powerful tool for the development of biomarkers for responses to specific challenges on the community (Oresic *et al*, 2006). In this study analysis of dual species biofilms by CLPP revealed that with the introduction of a new nutrient source (artificial GCF) and microaerophilic conditions, different patterns of substrate utilisation were observed. The highest levels of utilisation of carboxylic acids, nucleosides and sugar phosphates were observed with this change in conditions, indicating that responses such as these could be observed as markers of key changes in the microbial community. Microarray technologies focusing on the up and down-regulation of metabolic genes at different stages of biofilm development would also be a useful tool for characterizing changes in the microbial community. For example, Dennis *et al*, (2003) developed a DNA microarray to monitor the expression of bacterial metabolic genes within mixed microbial communities and concluded that microarrays

would be useful tools for the detection of bacterial gene expression in complex microbial systems.

As effective agents exist that reduce plaque accumulation it could be argued that it is not necessary to develop new treatments. However, many of these standard treatments such as chlorhexidine and tetracycline have a broad-spectrum action which not only decreases the number of pathogenic bacteria but also the commensal microbiota associated with oral health. Hence, the repeated or prolonged use of such agents could permanently affect the composition of the oral microbiota. Furthermore, they also have side effects on the host such as tooth staining (Sanchez *et al*, 2004; Jenkins *et al*, 1989). The aim with the development of new treatments is to use agents with a more selective mode of action, targeting species that are associated with disease whilst maintaining species associated with oral health. The use of models such as the one developed in this study, where experiments can be carried out longitudinally, has the potential to allow long term assessment of different agents on the microbial community. Treatments for prolonged use, with fewer side effects on the patient need to be assessed for their antimicrobial activity and the use of models such as this one are essential for initial assessment before reaching the expensive clinical trial stage.

This study has demonstrated that the *in vitro* model developed for gingivitis can successfully be applied to the assessment of microbial communities developing on dental materials releasing antibacterial agents. The agents used in this study were known to be effective against oral biofilm formation from previous studies (Pratten *et al*, 1998b; Ready *et al*, 2002) when applied topically. Targeting bacteria during plaque accumulation is the most effective method of controlling plaque-related diseases as the

bacteria are in a state when they are most likely to be susceptible to antimicrobial and anti-plaque agents. Indeed, the communities that developed in the CDFF model were most susceptible at the initial development stage. Interestingly, whilst the total bacterial numbers did increase over time, the presence of species associated with gingivitis such as *A. naeslundii* and *Prevotella* spp. were restricted when compared to communities that developed in the absence of these agents. *Fusobacterium* spp., which play a key role in the attachment of other species, including periodontal pathogens, were also reduced in these communities.

When treating periodontal diseases active agents should prevent biofilm formation without affecting the biological equilibrium within the oral cavity (Baehni & Takeuchi, 2003). This model could also be used to test novel approaches to the prevention of periodontal diseases which may be more suitable for long term use. Antibacterial agents derived from natural products are ideal candidates for such use. For example, plant extracts such as sanguinarine have been shown to significantly reduce gingivitis (Gonzalez-Begne *et al*, 2001), green tea catechins have been shown to be bactericidal against *P. gingivalis* and *Prevotella* spp. and to improve periodontal status (Hirasawa *et al*, 2002), whilst high molecular weight cranberry extract has been shown to reduce damage from periodontitis (Bodet *et al*, 2007).

A model is a simplified version of the environment it is attempting to replicate. The main value in such studies is to link specific environmental factors to changes in the community by having a degree of control over these factors which is not possible in the natural environment. The findings of this study have allowed specific changes in the oral microbial community to be linked to the presence of nutrients associated with

gingival inflammation and reduced oxygen availability in a model environment for the first time. This model was then used successfully to characterize changes in the microbial community associated with the presence of different antimicrobial agents highlighting the value of models such as this in the initial assessment of novel anti-plaque agents.

The model and assessment techniques developed here would be a valuable tool for testing potential agents for the control of plaque accumulation which would be the main focus of future research leading on from this study. As increased proportions of specific species are implicated in periodontal disease development and severity the qPCR primers developed for this study could also be useful for the rapid assessment of patient plaque samples. As this study has shown that changes in nutrient sources and atmospheric changes were influential on the microbial composition of microcosm communities future work could investigate the influence of other environmental parameters such as temperature and pH which are known to fluctuate with the development of periodontal diseases. Further characterization of the uncultivable portion of the biofilm communities developed in the CDFF using newly developed methodologies could also be another direction for future research. Indeed, models such as the one developed in this study are ideal for the assessment of new techniques which aim to characterize changes in the microbial community associated with the development of periodontal diseases, focussing on key changes in the microcosm environment as a whole, in factors such as substrate utilization and metabolite production, which could then be linked back to changes in specific species and genera using qPCR.

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0099-2240/06/$08.00+0 doi:10.1128/AEM.72.5.3678–3684.2006

Modeling Shifts in Microbial Populations Associated with Health or Disease

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Received 3 October 2005/Accepted 26 February 2006

JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2007, p. 3072–3076 Vol. 45, No. 9
0095-1137/07/$08.00+0 doi:10.1128/JCM.01131-07

Use of Quantitative PCR and Culture Methods To Characterize Ecological Flux in Bacterial Biofilms[▽]

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Received 6 June 2007/Accepted 21 June 2007